CONSENSUS/ANCESTRAL IMMUNOGENS

This application claims priority from Prov.
Appln. No. 60/503,460, filed September 17, 2003, and
Prov. Appln. No. 60/604,722, filed August 27, 2004,
5 the entire contents of which are incorporated herein
by reference.

TECHNICAL FIELD

The present invention relates, in general, to
an immunogen and, in particular, to an immunogen for
10 inducing antibodies that neutralize a wide spectrum
of HIV primary isolates and/or to an immunogen that
induces a T cell immune response. The invention
also relates to a method of inducing anti-HIV
antibodies, and/or to a method of inducing a T cell
15 immune response, using such an immunogen. The
invention further relates to nucleic acid sequences
encoding the present immunogens.

BACKGROUND

The high level of genetic variability of HIV-1
20 has presented a major hurdle for AIDS vaccine
development. Genetic differences among HIV-1 groups
M, N, and O are extensive, ranging from 30% to 50%
in *gag* and *env* genes, respectively (Gurtler et al,
J. Virol. 68:1581-1585 (1994), Vanden Haesevelde et
25 al, J. Virol. 68:1586-1596 (1994), Simon et al, Nat.
Med. 4:1032-1037 (1998), Kuiken et al, Human

retroviruses and AIDS 2000: a compilation and
analysis of nucleic acid and amino acid sequences
(Theoretical Biology and Biophysics Group, Los
Alamos National Laboratory, Los Alamos, New

5   Mexico)). Viruses within group M are further
classified into nine genetically distinct subtypes
(A-D, F-H, J and K) (Kuiken et al, Human
retroviruses and AIDS 2000: a compilation and
analysis of nucleic acid and amino acid sequences

10   (Theoretical Biology and Biophysics Group, Los
Alamos National Laboratory, Los Alamos, New Mexico,
Robertson et al, Science 288:55-56 (2000), Robertson
et al, Human retroviruses and AIDS 1999: a
compilation and analysis of nucleic acid and amino

15   acid sequences, eds. Kuiken et al (Theoretical
Biology and Biophysics Group, Los Alamos National
Laboratory, Los Alamos, New Mexico), pp. 492-505
(2000)). With the genetic variation as high as 30%
in env genes among HIV-1 subtypes, it has been

20   difficult to consistently elicit cross-subtype T and
B cell immune responses against all HIV-1 subtypes.
HIV-1 also frequently recombines among different
subtypes to create circulating recombinant forms
(CRFs) (Robertson et al, Science 288:55-56 (2000),

25   Robertson et al, Human retroviruses and AIDS 1999: a
compilation and analysis of nucleic acid and amino
acid sequences, eds. Kuiken et al (Theoretical
Biology and Biophysics Group, Los Alamos National
Laboratory, Los Alamos, New Mexico), pp. 492-505

30   (2000), Carr et al, Human retroviruses and AIDS
1998: a compilation and analysis of nucleic acid and

amino acid sequences, eds. Korber et al (Theoretical
Biology and Biophysics Group, Los Alamos National
Laboratory, Los Alamos, New Mexico), pp. III-10-III-
19 (1998)). Over 20% of HIV-1 isolates are

5   recombinant in geographic areas where multiple
subtypes are common (Robertson et al, Nature
374:124-126 (1995), Cornelissen et al, J. virol.
70:8209-8212 (1996), Dowling et al, AIDS 16:1809-
1820 (2002)), and high prevalence rates of

10   recombinant viruses may further complicate the
design of experimental HIV-1 immunogens.

To overcome these challenges in AIDS vaccine
development, three computer models (consensus,
ancestor and center of the tree) have been used to

15   generate centralized HIV-1 genes to (Gaschen et al,
Science 296:2354-2360 (2002), Gao et al, Science
299:1517-1518 (2003), Nickle et al, Science
299:1515-1517 (2003), Novitsky et al, J. Virol.
76:5435-5451 (2002), Ellenberger et al, Virology

20   302:155-163 (2002), Korber et al, Science 288:1789-
1796 (2000)). The biology of HIV gives rise to
star-like phylogenies, and as a consequence of this,
the three kinds of sequences differ from each other
by 2 - 5% (Gao et al, Science 299:1517-1518 (2003)).

25   Any of the three centralized gene strategies will
reduce the protein distances between immunogens and
field virus strains. Consensus sequences minimize
the degree of sequence dissimilarity between a
vaccine strain and contemporary circulating viruses

30   by creating artificial sequences based on the most
common amino acid in each position in an alignment

(Gaschen et al, Science 296:2354-2360 (2002)).
Ancestral sequences are similar to consensus
sequences but are generated using maximum-likelihood
phylogenetic analysis methods (Gaschen et al,

5   Science 296:2354-2360 (2002), Nickle et al, Science
299:1515-1517 (2003)). In doing so, this method
recreates the hypothetical ancestral genes of the
analyzed current wild-type sequences (Figure 26).
Nickle et al proposed another method to generate

10   centralized HIV-1 sequences, center of the tree
(COT), that is similar to ancestral sequences but
less influenced by outliers (Science 299:1515-1517
(2003)).

       The present invention results, at least in

15   part, from the results of studies designed to
determine if centralized immunogens can induce both
T and B cell immune responses in animals. These
studies involved the generation of an artificial
group M consensus env gene (CON6), and construction

20   of DNA plasmids and recombinant vaccinia viruses to
express CON6 envelopes as soluble gp120 and gp140CF
proteins. The results demonstrate that CON6 Env
proteins are biologically functional, possess
linear, conformational and glycan-dependent epitopes

25   of wild-type HIV-1, and induce cytokine-producing T
cells that recognize T cell epitopes of both HIV
subtypes B and C. Importantly, CON6 gp120 and
gp140CF proteins induce antibodies that neutralize
subsets of subtype B and C HIV-1 primary isolates.

30   The iterative nature of study of the
centralized HIV-1 gene approach is derived from the

rapidly expanding evolution of HIV-1 sequences, and
the fact that sequences collected in the HIV
sequence database (that is, the Los Alamos National
Database) are continually being updated with new
sequences each year. The CON6 gp120 envelope gene
derives from Year 1999 Los Alamos National Database
sequences, and Con-S derives from Year 2000 Los
Alamos National Database sequences. In addition,
CON6 has Chinese subtype C V1, V2, V4, and V5 Env
sequences, while Con-S has all group M consensus Env
constant and variable regions, that have been
shortened to minimal-length variable loops. Codon-
optimized genes for a series of Year 2003 group M
and subtype consensus sequences have been designed,
as have a corresponding series of wild-type HIV-1
Env genes for comparison, for use in inducing
broadly reactive T and B cell responses to HIV-1
primary isolates.

SUMMARY OF THE INVENTION

The present invention relates to an immunogen
for inducing antibodies that neutralize a wide
spectrum of HIV primary isolates and/or to an
immunogen that induces a T cell immune response, and
to nucleic acid sequences encoding same. The
invention also relates to a method of inducing anti-
HIV antibodies, and/or to a method of inducing a T
cell immune response, using such an immunogen.

Objects and advantages of the present invention
will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

     Figures 1A-1D: Generation and expression of the group M consensus *env* gene (CON6). The complete amino acid sequence of CON6 gp160 is shown.

5 (Fig. 1A) The five regions from the wild-type CRF08_BC (98CN006) *env* gene are indicated by underlined letters. Variable regions are indicated by brackets above the sequences. Potential N-liked glycosylation sites are highlighted with bold-faced

10 letters. (Fig. 1B) Constructs of CON6 gp120 and gp140CF. CON6 gp120 and gp140CF plasmids were engineered by introducing a stop codon after the gp120 cleavage site or before the transmembrane domain, respectively. The gp120/gp41 cleavage site

15 and fusion domain of gp41 were deleted in the gp140CF protein. (Fig.1C) Expression of CON6 gp120 and gp140CF. CON6 gp120 and gp140CF were purified from the cell culture supernatants of rVV-infected 293T cells with *galanthus Nivalis argarose* lectin

20 columns. Both gp120 and gp140CF were separated on a 10% SDS-polyarylamide gel and stained with Commassie blue. (Fig. 1D.) CON6 *env* gene optimized based on codon usage for highly expressed human genes.

     Figures 2A-2E. Binding of CON6 gp120 gp140 CF

25 to soluble CD4 (sCD4) and anti-Env mAbs. (Figs. 2A-2B) Each of the indicated mabs and sCD4 was covalently immobilized to a CM5 sensor chip (BIAcore) and CON6 gp120 (Fig. 2A) or gp140CF (Fig.

2B) (100 μg/ml and 300 μg/ml, respectively) were injected over each surface. Both gp120 and gp140CF proteins reacted with each anti-gp120 mabs tested except for17b mab, which showed negligible binding
5   to both CON6 gp120 and gp140CF. To determine induction of 17b mab binding to CON6 gp120 and gp140CF, CON6 gp120 (Fig. 2C) or gp140CF (Fig. 2D) proteins were captured (400-580 RU) on individual flow cells immobilized with sCD4 or mabs A32 or T8.
10 Following stabilization of each of the surface, mAb 17b was injected and flowed over each of the immobilized flow cells. Overlay of curves show that the binding of mab 17b to CON6 Env proteins was markedly enhanced on both sCD4 and mab A32 surfaces
15 but not on the T8 surface (Figs. 2C-2D). To determine binding of CON6 gp120 and gp140CF to human mabs in ELISA, stock solutions of 20μg/ml of mabs 447, F39F, A32, IgG1b12 and 2F5 on CON6 gp120 and gp140CF were tittered (Fig. 2E). Mabs 447 (V3),
20 F39F (V3) A32 (gp120) and IgG1b12 (CD4 binding site) each bound to both CON6 gp120 and 140 well, while 2F5 (anti-gp41 ELDKWAS) only bound gp140CF. The concentration at endpoint titer on gp120 for mab 447 and F39F binding was <0.003 μg/ml and 0.006 μg/ml,
25 respectively; for mab A32 was <0.125 μg/ml; for IgG1b12 was <0.002 μg/ml; and for 2F5 was 0.016 μg/ml.

Figures 3A and 3B. Infectivity and coreceptor usage of CON6 envelope. (Fig. 3A) CON6 and control

env plasmids were cotransfected with HIV-1/SG3Δenv
backbone into human 293T cells to generate Env-
pseudovirions.   Equal amounts of each pseudovirion
(5 ng p24) were used to infect JC53-BL cells.   The
5   infectivity was determined by counting the number of
blue cells (infectious units, IU) per microgram of
p24 of pseudovirons (IU/µg p24) after staining the
infected cells for β-gal expression.   (Fig. 3B)
Coreceptor usage of the CON6 *env* gene was determined
10   on JC53BL cells treated with AMD3100 and/or TAK-799
for 1 hr (37°C) then infected with equal amounts of
p24 (5 ng) of each Env-pseudovirion.   Infectivity in
the control group (no blocking agent) was set as
100%.   Blocking efficiency was expressed as the
15   percentage of IU from blocking experiments compared
to those from control cultures without blocking
agents.   Data shown are mean ± SD.

Figure 4.   Western blot analysis of multiple
subtype Env proteins against multiple subtype
20   antisera.   Equal amount of Env proteins (100 ng)
were separated on 10% SDS-polyacrylamide gels.
Following electrophoresis, proteins were transferred
to Hybond ECL nitrocellulose membranes and reacted
with sera from HIV-1 infected patients (1:1,000) or
25   guinea pigs immunized with CON6 gp120 DNA prime, rVV
boost (1:1,000).   Protein-bound antibody was probed
with fluorescent-labeled secondary antibodies and
the images scanned and recorded on an infrared
imager Odyssey (Li-Cor, Lincoln, NE).   Subtypes are

indicated by single-letters after Env protein and
serum IDs. Four to six sera were tested for each
subtype, and reaction patterns were similar among
all sera from the same subtype. One representative
5   result for each subtype serum is shown.

Figure 5. T cell immune responses induced by
CON6 Env immunogens in mice. Splenocytes were
isolated from individual immunized mice (5
mice/group). After splenocytes were stimulated *in
10   vitro* with overlapping Env peptide pools of CON6
(black column), subtype B (hatched column), subtype
C (white column), and medium (no peptide; gray
column), INF-γ producing cells were determined by
the ELISPOT assay. T cell IFN-γ responses induced
15   by either CON6 gp120 or gp140CF were compared to
those induced by subtype specific Env immunogens
(JRFL and 96ZM651). Total responses for each
envelope peptide pool are expressed as SFCs per
million splenocytes. The values for each column are
20   the mean ± SEM(of IFN-γ SFCs (n=5 mice/group).

Figures 6A-6E. Construction of codon usage
optimized subtype C ancestral and consensus envelope
genes (Figs. 6A and 6B, respectively). Ancestral
and consensus amino acid sequences (Figs. 6C and 6D,
25   respectively) were transcribed to mirror the codon
usage of highly expressed human genes. Paired
oligonucleotides (80-mers) overlapping by 20 bp were
designed to contain 5' invariant sequences including

the restriction enzyme sites EcoRI, BbsI, Bam HI and
BsmBI. BbsI and BsmBI are Type II restriction
enzymes that cleave outside of their recognition
sequences. Paired oligomers were linked
5   individually using PCR and primers complimentary to
the 18 bp invariant sequences in a stepwise fashion,
yielding 140bp PCR products. These were subcloned
into pGEM-T and sequenced to confirm the absence of
inadvertant mutations/deletions. Four individual
10 pGEM-T subclones containing the proper inserts were
digested and ligated together into pcDNA3.1. Multi-
fragment ligations occurred repeatly amongst groups
of fragments in a stepwise manner from the 5' to the
3' end of the gene until the entire gene was
15 reconstructed in pcDNA3.1. (See schematic in Fig.
6E.)

    Figure 7. JC53-BL cells are a derivative of
HeLa cells that express high levels of CD4 and the
HIV-1 coreceptors CCR5 and CXCR4. They also contain
20 the reporter cassettes of luciferase and β-
galactosidase that are each expressed from an HIV-1
LTR. Expression of the reporter genes is dependent
on production of HIV-1 Tat. Briefly, cells are
seeded into 24 or 96-well plates, incubated at 37°C
25 for 24 hours and treated with DEAE-Dextran at 37°C
for 30 minutes. Virus is serially diluted in 1%
DMEM, added to the cells incubating in DEAE-Dextran,
and allowed to incubate for 3 hours at 37°C after
which an additional cell media is added to each

well.   Following a final 48-hour incubation at 37°C,
cells are either fixed, stained using X-Gal to
visualize β-galactosidase expressing blue foci or
frozen-thawed three times to measure luciferase

5   activity.

       Figure 8.   Sequence alignment of subtype C
ancestral and consensus env genes.   Alignment of the
subtype C ancestral (bottom line) and consensus (top
line) env sequences showing a 95.5% sequence

10   homology; amino acid sequence differences are
indicated. One noted difference is the addition of a
glycosylation site in the C ancestral env gene at
the base of the V1 loop. A plus sign indicates a
within-class difference of amino acid at the

15   indicated position; a bar indicates a change in the
class of amino acid. Potential N-glycosylation sites
are marked in blue. The position of truncation for
the gp140 gene is also shown.

       Figure 9.   Expression of subtype C ancestral

20   and consensus envelopes in 293T cells.   Plasmids
containing codon-optimized gp160, gp140, or gp120
subtype C ancestral and consensus genes were
transfected into 293T cells, and protein expression
was examined by Western Blot analysis of cell

25   lysates.   48-hours post-transfection, cell lysates
were collected, total protein content determined by
the BCA protein assay, and 2 μg of total protein was
loaded per lane on a 4-20% SDS-PAGE gel.   Proteins

                                11

were transferred to a PVDF membrane and probed with
HIV-1 plasma from a subtype C infected patient.

    Figures 10A and 10B. Fig. 10A. *Trans*
complementation of *env*-deficient HIV-1 with codon-
5 optimized subtype C ancestral and consensus gp160
and gp140. Plasmids containing codon-optimized,
subtype C ancestral or consensus *gp160* or *gp140*
genes were co-transfected into 293T cells with an
HIV-1/SG3Δ*env* provirus. 48 hours post-transfection
10 cell supernatants containing pseudotyped virus were
harvested, clarified by centrifugation, filtered
through at 0.2μM filter, and pelleted through a 20%
sucrose cushion. Quantification of p24 in each
virus pellet was determined using the Coulter HIV-1
15 p24 antigen assay; 25ng of p24 was loaded per lane
on a 4-20% SDS-PAGE gel for particles containing a
codon-optimized envelope. 250ng of p24 was loaded
per lane for particles generated by co-transfection
of a rev-dependent wild-type subtype C 96ZAM651*env*
20 gene. Differences in the amount of p24 loaded per
lane were necessary to ensure visualization of the
rev-dependent envelopes by Western Blot. Proteins
were transferred to a PVDF membrane and probed with
pooled plasma from HIV-1 subtype B and subtype C
25 infected individuals. Fig. 10B. Infectivity of
virus particles containing subtype C ancestral and
consensus envelope glycoproteins. Infectivity of
pseudotyped virus containing ancestral or consensus
gp160 or *gp140* envelope was determined using the

JC53-BL assay. Sucrose cushion purified virus
particles were assayed by the Coulter p24 antigen
assay, and 5-fold serial dilutions of each pellet
were incubated with DEAE-Dextran treated JC53-BL
5   cells. Following a 48-hour incubation period, cells
were fixed and stained to visualize β-galactosidase
expressing cells. Infectivity is represented as
infectious units per ng of p24 to normalize for
differences in the concentration of the input
10   pseudovirions.

     Figure 11. Co-receptor usage of subtype C
ancestral and consensus envelopes. Pseudotyped
particles containing ancestral or consensus envelope
were incubated with DEAE-Dextran treated JC53-BL
15   cells in the presence of AMD3100 (a specific
inhibitor of CXCR4), TAK779 (a specific inhibitor of
CCR5), or AMD3000+TAK779 to determine co-receptor
usage. NL4.3, an isolate known to utilize CXCR4,
and YU-2, a known CCR5-using isolate, were included
20   as controls.

     Figures 12A-12C. Neutralization sensitivity of
subtype C ancestral and consensus envelope
glycoproteins. Equivalent amounts of pseudovirions
containing the ancestral, consensus or 96ZAM651
25   *gp160* envelopes (1,500 infectious units) were pre-
incubated with a panel of plasma samples from HIV-1
subtype C infected patients and then added to the
JC53-BL cell monolayer in 96-well plates. Plates

13

were cultured for two days and luciferase activity
was measured as an indicator of viral infectivity.
Virus infectivity is calculated by dividing the
luciferase units (LU) produced at each concentration
5   of antibody by the LU produced by the control
infection. The mean 50% inhibitory concentration
(IC_{50}) and the actual % neutralization at each
antibody dilution are then calculated for each
virus. The results of all luciferase experiments
10   are confirmed by direct counting of blue foci in
parallel infections.

Figures 13A-13F. Protein expression of
consensus subtype C Gag (Fig. 13A) and Nef (Fig.
13B) following transfection into 293T cells.
15   Consensus subtype C Gag and Nef amino acid sequences
are set forth in Figs. 13C and 13D, respectively,
and encoding sequences are set forth in Figs. 13E
and 13F, respectively.

Figures 14A-14C. Figs. 14A and 14B show the
20   Con-S Env amino acid sequence and encoding sequence,
respectively. Fig. 14C shows expression of Group M
consensus Con-S Env proteins using an *in vitro*
transcription and translation system.

Figures 15A and 15B. Expression of Con-S *env*
25   gene in mammalian cells. (Fig. 15A - cell lysate,
Fig. 15B - supernatant.)

Figures 16A and 16B.   Infectivity (Fig. 16A)
and coreceptor usage (Fig. 16B) of CON6 and Con-S
env genes.

5   Figures 17A-17C.   Env protein incorporation in
CON6 and Con-S Env-pseudovirions.   (Fig. 17A –
lysate, Fig. 17B – supernatant, Fig. 17C pellet.)

Figures 18A-18D.   Figs. 18A and 18B show
subtype A consensus Env amino acid sequence and
nucleic acid sequence encoding same, respectively.
10   Figs. 18C and 18D show expression of A.con *env* gene
in mammalian cells (Fig. 18C – cell lysate, Fig. 18D
– supernatant).

Figures 19A-19H.   M.con.gag (Fig. 19A),
M.con.pol (Fig. 19B), M.con.nef (Fig. 19C) and
15   C.con.pol (Fig. 19D) nucleic acid sequences and
corresponding encoded amino acid sequences (Figs.
19E-19H, respectively).

Figures 20A-20D.   Subtype B consensus *gag* (Fig.
20A) and *env* (Fig.20B) genes.   Corresponding amino
20   acid sequences are shown in Figs. 20C and 20D.

Figure 21.   Expression of subtype B consensus
env and *gag* genes in 293T cells.   Plasmids
containing codon-optimized subtype B consensus
gp160, *gp140*, and *gag* genes were transfected into
25   293T cells, and protein expression was examined by

Western Blot analysis of cell lysates. 48-hours
post-transfection, cell lysates were collected,
total protein content determined by the BCA protein
assay, and 2 μg of total protein was loaded per lane
5   on a 4-20% SDS-PAGE gel. Proteins were transferred
to a PVDF membrane and probed with serum from an
HIV-1 subtype B infected individual.

Figure 22. Co-receptor usage of subtype B
consensus envelopes. Pseudotyped particles
10 containing the subtype B consensus gp160 Env were
incubated with DEAE-Dextran treated JC53-BL cells in
the presence of AMD3100 (a specific inhibitor of
CXCR4), TAK779 (a specific inhibitor of CCR5), and
AMD3000+TAK779 to determine co-receptor usage.
15 NL4.3, an isolate known to utilize CXCR4 and YU-2, a
known CCR5-using isolate, were included as controls.

Figures 23A and 23B. *Trans* complementation of
env-deficient HIV-1 with codon-optimized subtype B
consensus *gp160* and *gp140* genes. Plasmids
20 containing codon-optimized, subtype B consensus
gp160 or *gp140* genes were co-transfected into 293T
cells with an HIV-1/SG3Δenv provirus. 48-hours
post-transfection cell supernatants containing
pseudotyped virus were harvested, clarified in a
25 tabletop centrifuge, filtered through a 0.2μM
filter, and pellet through a 20% sucrose cushion.
Quantification of p24 in each virus pellet was
determined using the Coulter HIV-1 p24 antigen

assay; 25 ng of p24 was loaded per lane on a 4-20%
SDS-PAGE gel. Proteins were transferred to a PVDF
membrane and probed with anti-HIV-1 antibodies from
infected HIV-1 subtype B patient serum. *Trans*
5 complementation with a rev-dependent NL4.3 *env* was
included for control. Figure 23B. Infectivity of
virus particles containing the subtype B concensus
envelope. Infectivitiy of pseudotyped virus
containing consensus B gp160 or gp140 was determined
10 using the JC53-BL assay. Sucrose cushion purified
virus particles were assayed by the Coulter p24
antigen assay, and 5-fold serial dilutions of each
pellet were incubated with DEAE-Dextran treated
JC53-BL cells. Following a 48-hour incubation
15 period, cells were fixed and stained to visualize β-
galactosidase expressing cells. Infectivity is
expressed as infectious units per ng of p24.

Figures 24A-24D. Neutralization sensitivity of
virions containing subtype B consensus gp160
20 envelope. Equivalent amounts of pseudovirions
containing the subtype B consensus or NL4.3 Env
(gp160) (1,500 infectious units) were preincubated
with three different monoclonal neutralizing
antibodies and a panel of plasma samples from HIV-1
25 wubtype B infected individuals, and then added to
the JC53-BL cell monolayer in 96-well plates.
Plates were cultured for two days and luciferase
activity was measured as an indicator of viral
infectivity. Virus infectivity was calculated by

dividing the luciferase units (LU) produced at each
concentration of antibody by the LU produced by the
control infection. The mean 50% inhibitory
concentration (IC_{50}) and the actual % neutralization
5   at each antibody dilution were then calculated for
each virus. The results of all luciferase
experiments were confirmed by direct counting of
blue foci in parallel infections. Fig. 24A.
Neutralization of Pseudovirions containing Subtype B
10   consensus Env (gp160). Fig. 24B. Neutralization of
Pseudovirions containing NL4.3 Env (gp160).
Fig. 24C. Neutralization of Pseudovirions containing
Subtype B consensus Env (gp160). Fig. 24D.
Neutralization of Pseudovirions containing NL4.3 Env
15   (gp160).

     Figures 25A and 25B. Fig. 25A. Density and p24
analysis of sucrose gradient fractions. 0.5ml
fractions were collected from a 20-60% sucrose
gradient. Fraction number 1 represents the most
20   dense fraction taken from the bottom of the gradient
tube. Density was measured with a refractometer and
the amount of p24 in each fraction was determined by
the Coulter p24 antigen assay. Fractions 6-9, 10-
15, 16-21, and 22-25 were pooled together and
25   analyzed by Western Blot. As expected, virions
sedimented at a density of 1.16-1.18 g/ml.
Fig. 25B. VLP production by co-transfection of
subtype B consensus *gag* and *env* genes. 293T cells
were co-transfected with subtype B consensus *gag* and

env genes. Cell supernatants were harvested 48-
hours post-transfection, clarified through at 20%
sucrose cushion, and further purified through a 20-
60% sucrose gradient. Select fractions from the

5   gradient were pooled, added to 20ml of PBS, and
centrifuged overnight at 100,000 x g. Resuspended
pellets were loaded onto a 4-20% SDS-PAGE gel,
proteins were transferred to a PVDF membrane, and
probed with plasma from an HIV-1 subtype B infected

10   individual.

     Figures 26A and 26B. Fig. 26A. 2000 Con-S
140CFI.ENV. Fig. 26B. Codon-optimized Year 2000
Con-S 140CFI.seq.

     Figure 27. Individual C57BL/6 mouse T cell

15   responses to HIV-1 envelope peptides. Comparative
immunogenicity of CON6 gp140CFI and Con-S gp140CFI
in C57BL/C mice. Mice were immunized with either
HIV5305 (Subtype A), 2801 (Subtype B), CON6 or Con-S
Envelope genes in DNA prime, rVV boost regimens, 5

20   mice per group. Spleen cells were assayed for IFN-γ
spot-forming cells 10 days after rVV boost, using
mixtures of overlapping peptides from Envs of HIV-1
UG37(A), MN(B), Ch19(C), 89.6(B) SF162(B) or no
peptide negative control.

25   Figures 28A-28C. Fig. 28A. Con-B 2003 Env. pep
(841 a.a.). Amino acid sequence underlined is the
fusion domain that is deleted in 140CF design and

the "W" underlined is the last amino acid at the
C-terminus, all amino acids after the "W" are
deleted in the 140CF design. Fig. 28B. Con-B-
140CF.pep (632 a.a.). Amino acids in bold identify
5   the junction of the deleted fusion cleavage site.
Fig. 28C. Codon-optimized Con-B 140CF.seq
(1927 nt.).

Figures 29A-29C. Fig. 29A. CON_OF_CONS-2003
(829 a.a.). Amino acid sequence underlined is the
10   fusion domain that is deleted in 140CF design and
the "W" underlined is the last amino acid at the
C-terminus, all amino acids after the "W" are
deleted in the 140CF design. Fig. 29B. ConS-2003
140CF.pep (620 a.a.). Amino acids in bold identify
15   the junction of the deleted fusion cleavage site.
Fig. 29C. CODON-OPTIMIZED ConS-2003 140CF.seq (1891
nt.).

Figures 30A-30C. Fig. 30A. CONSENSUS_A1-2003
(845 a.a.). Amino acid sequence underlined is the
20   fusion domain that is deleted in 140CF design and
the "W" underlined is the last amino acid at the
C-terminus, all amino acids after the "W" are
deleted in the 140CF design. Fig. 30B. Con-A1-2003
140CF.pep (629 a.a.). Amino acids in bold identify
25   the junction of the deleted fusion cleavage site.
Fig. 30C. CODON-OPTIMIZED Con-A1-2003.seq.

Figures 31A-31C. Fig. 31A. CONSENSUS_C-2003
(835 a.a.). Amino acid sequence underlined is the
fusion domain that is deleted in 140CF design and
the "W" underlined is the last amino acid at the
5   C-terminus, all amino acids after the "W" are
deleted in the 140CF design. Fig. 31B. Con-C 2003
140CF.pep (619 a.a.). Amino acids in bold identify
the junction of the deleted fusion cleavage site.
Fig. 31C. CODON-OPTIMIZED Con-C-2003 (140 CF (1,888
10   nt.).

Figures 32A-32C. Fig. 32A. CONSENSUS_G-2003
(842 a.a.). Amino acid sequence underlined is the
fusion domain that is deleted in 140CF design and
the "W" underlined is the last amino acid at the
15   C-terminus, all amino acids after the "W" are
deleted in the 140CF design. Fig. 32B. Con-G-2003
140CF.pep (626 a.a.). Amino acids in bold identify
the junction of the deleted fusion cleavage site.
Fig. 32C. CODON-OPTIMIZED Con-G-2003.seq.

20   Figures 33A-33C. Fig. 33A. CONSENSUS_01_AE-
2003 (854 a.a.). Amino acid sequence underlined is
the fusion domain that is deleted in 140CF design
and the "W" underlined is the last amino acid at
the C-terminus, all amino acids after the "W" are
25   deleted in the 140CF design. Fig. 33B. Con-AE01-
2003 140CF.pep (638 a.a.). Amino acids in bold
identify the junction of the deleted fusion cleavage

site.   Fig. 33C.   CODON-OPTIMIZED Con-AE01-2003.seq.
(1945 nt.).

     Figures 34A-34C.   Fig. 34A.   Wild-type subtype
A Env.   00KE_MSA4076-A (Subtype A, 891 a.a.).   Amino
5   acid sequence underlined is the fusion domain that
is deleted in 140CF design and the "W" underlined
is the last amino acid at the C-terminus, all amino
acids after the "W" are deleted in the 140CF design.
Fig. 34B.   00KE_MSA4076-A 140CF.pep (647 a.a.).
10 Amino acids in bold identify the junction of the
deleted fusion cleavage site.   Fig. 34C.   CODON-
OPTIMIZED 00KE_MSA4076-A 140CF.seq. (1972 nt.).

     Figures 35A-35C.   Fig. 35A.   Wild-type subtype
B.   QH0515.1g gp160 (861 a.a.).   Amino acid sequence
15 underlined is the fusion domain that is deleted in
140CF design and the "W" underlined is the last
amino acid at the C-terminus, all amino acids after
the "W" are deleted in the 140CF design.   Fig. 35B.
QH0515.1g 140CF (651 a.a.).   Amino acids in bold
20 identify the junction of the deleted fusion cleavage
site.   Fig. 35C.   CODON-OPTIMIZED QH0515.1g
140CF.seq (1984 nt.).

     Figures 36A-36C.   Fig. 36A.   Wild-type subtype
C.   DU123.6 gp160 (854 a.a.).   Amino acid sequence
25 underlined is the fusion domain that is deleted in
140CF design and the "W" underlined is the last
amino acid at the C-terminus, all amino acids after

the "W" are deleted in the 140CF design. Fig. 36B.
DU123.6 140CF (638 a.a.). Amino acids in bold
identify the junction of the deleted fusion cleavage
site. Fig. 36C. CODON-OPTIMIZED DU123.6 140CF.seq
5   (1945 nt.).

     Figures 37A-37C. Fig. 37A. Wild-type subtype
CRF01_AE. 97CNGX2F-AE (854 a.a.). Amino acid
sequence underlined is the fusion domain that is
deleted in 140CF design and the "W" underlined is
10   the last amino acid at the C-terminus, all amino
acids after the "W" are deleted in the 140CF design.
Fig. 37B. 97CNGX2F-AE 140CF.pep (629 a.a.). Amino
acids in bold identify the junction of the deleted
fusion cleavage site. Fig. 37C. CODON-OPTIMIZED
15   97CNGX2F-AE 140CF.seq (1921 nt.).

     Figures 38A-38C. Fig. 38A. Wild-type DRCBL-G
(854 a.a.). Amino acid sequence underlined is the
fusion domain that is deleted in 140CF design and
the "W" underlined is the last amino acid at the
20   C-terminus, all amino acids after the "W" are
deleted in the 140CF design. Fig. 38B. DRCBL-G
140CF.pep (630 a.a.). Amino acids in bold identify
the junction of the deleted fusion cleavage site.
Fig. 38C. CODON-OPTIMIZED DRCBL-G 140CF.seq (1921
25   nt.).

Figures 39A and 39B. Fig. 39A. 2003 Con-S
Env. Fig. 39B. 2003 Con-S Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 40A and 40B. Fig. 40A. 2003 M.
5   Group.Anc Env. Fig. 40B. 2003 M. Group.anc
Env.seq.opt. (Seq.opt. = codon optimized encoding
sequence.)

Figures 41A and 41B. Fig. 41A. 2003 CON_A1
Env. Fig. 41B. 2003 CON_A1 Env.seq.opt.
10   (Seq.opt. = codon optimized encoding sequence.)

Figures 42A and 42B. Fig. 42A. 2003 A1.Anc
Env. Figs. 42B. 2003 A1.anc Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 43A and 43B. Fig. 43A. 2003 CON_A2
15   Env. Fig. 43B. 2003 CON_A2 Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 44A and 44B. Fig. 44A. 2003 CON_B
Env. Fig. 44B. 2003 CON_B Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

20   Figures 45A and 45B. Fig. 45A. 2003 B.anc
Env. Figs. 45B. 2003 B.anc Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 46A and 46B. Fig. 46A. 2003 CON_C
Env. Fig. 46B. 2003 CON_C Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 47A and 47B. Fig. 47A. 2003 C.anc
5   Env. Fig. 47B. 2003 C.anc Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 48A and 48B. Fig. 48A. 2003 CON_D
Env. Fig. 48B. 2003 CON_D Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

10   Figures 49A and 49B. Fig. 49A. 2003 CON_F1
Env. Fig. 49B. 2003 CON_F1 Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 50A and 50B. Fig. 50A. 2003 CON_F2
Env. Fig. 50B. 2003 CON_F2 Env.seq.opt.
15 (Seq.opt. = codon optimized encoding sequence.)

Figures 51A and 51B. Fig. 51A. 2003 CON_G
Env. Fig. 51B. 2003 CON_G Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 52A and 52B. Fig. 52A. 2003 CON_H
20 Env. Fig. 52B. 2003 CON_H Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 53A and 53B. Fig. 53A. 2003 CON_01_AE
Env. Fig. 53B. 2003 CON_01_AE Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 54A and 54B. Fig. 54A. 2003 CON_02_AG
5   Env. Fig. 54B. 2003 CON_02_AG Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 55A and 55B. Fig. 55A. 2003 CON_03_AB
Env. Fig. 55B. 2003 CON_03_AB Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

10   Figures 56A and 56B. Fig. 56A. 2003
CON_04_CPX Env. Fig. 56B. 2003 CON_04_CPX
Env.seq.opt. (Seq.opt. = codon optimized encoding
sequence.)

Figures 57A and 57B. Fig. 57A. 2003
15   CON_06_CPX Env. Fig. 57B. 2003 CON_06_CPX
Env.seq.opt. (Seq.opt. = codon optimized encoding
sequence.)

Figures 58A and 58B. Fig. 58A. 2003 CON_08_BC
Env. Fig. 58B. 2003 CON_08_BC Env.seq.opt.
20   (Seq.opt. = codon optimized encoding sequence.)

Figures 59A and 59B. Fig. 59A. 2003 CON_10_CD
Env. Fig. 59B. 2003 CON_10_CD Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 60A and 60B. Fig. 60A. 2003
CON_11_CPX Env. Fig. 60B. 2003 CON_11_CPX
Env.seq.opt. (Seq.opt. = codon optimized encoding
sequence.)

5   Figures 61A and 61B. Fig. 61A. 2003 CON_12_BF
Env. Fig. 61B. 2003 CON_12_BF Env.seq.opt.
(Seq.opt. = codon optimized encoding sequence.)

Figures 62A and 62B. Fig. 62A. 2003 CON_14_BG
Env. Fig. 62B. 2003 CON_14_BG Env.seq.opt.
10   (Seq.opt. = codon optimized encoding sequence.)

Figures 63A and 63B. Fig. 63A. 2003_CON_S
gag.PEP. Fig. 63B. 2003_CON_S gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 64A and 64B. Fig. 64A.
15   2003_M.GROUP.anc gag.PEP. Fig. 64B.
2003_M.GROUP.anc gag.OPT. (OPT = codon optimized
encoding sequence.)

Figures 65A-65D. Fig. 65A. 2003_CON_A1
gag.PEP. Fig. 65B. 2003_CON_A1 gag.OPT. Fig. 65C.
20   2003_A1.anc gag.PEP. Fig. 65D. 2003_A1.anc
gag.OPT. (OPT = codon optimized encoding sequence.)

Figures 66A and 66B. Fig. 66A. 2003_CON_A2
gag.PEP. Fig. 66B. 2003_CON_A2 gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 67A-67D. Fig. 67A. 2003_CON_B

5   gag.PEP. Fig. 67B. 2003_CON_B gag.OPT. Fig. 67C.
2003_B.anc gag.PEP. Fig. 67D. 2003_B.anc gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 68A-68D. Fig. 68A. 2003_CON_C
gag.PEP. Fig. 68B. 2003_CON_C gag.OPT. Fig. 68C.

10 2003_C.anc.gag.PEP. Fig. 68D. 2003_C.anc.gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 69A and 69B. Fig. 69A. 2003_CON_D
gag.PEP. Fig. 69B. 2003_CON_D gag.OPT.
(OPT = codon optimized encoding sequence.)

15 Figures 70A and 70B. Fig. 70A. 2003_CON_F
gag.PEP. Fig. 70B. 2003_CON_F gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 71A and 71B. Fig. 71A. 2003_CON_G
gag.PEP. Fig. 71B. 2003_CON_G gag.OPT.

20 (OPT = codon optimized encoding sequence.)

Figures 72A and 72B. Fig. 72A. 2003_CON_H
gag.PEP. Fig. 72B. 2003_CON_H gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 73A and 73B. Fig. 73A. 2003_CON_K
gag.PEP. Fig. 73B. 2003_CON_K gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 74A and 74B. Fig. 74A. 2003_CON_01_AE
5   gag.PEP. Fig. 7B. 2003_CON_01_AE gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 75A and 75B. Fig. 75A. 2003_CON_02_AG
gag.PEP. Fig. 75B. 2003_CON_02_AG gag.OPT.
(OPT = codon optimized encoding sequence.)

10   Figures 76A and 76B. Fig. 76A.
2003_CON_03_ABG gag.PEP. Fig. 76B. 2003_CON_03_ABG
gag.OPT. (OPT = codon optimized encoding sequence.)

Figures 77A and 77B. Fig. 77A.
2003_CON_04_CFX gag.PEP. Fig. 77B. 2003 CON_04_CFX
15   gag.OPT. (OPT = codon optimized encoding sequence.)

Figures 78A and 78B. Fig. 78A.
2003_CON_06_CPX gag.PEP. Fig. 78B. 2003_CON_06_CPX
gag.OPT. (OPT = codon optimized encoding sequence.)

Figures 79A and 79B. Fig. 79A. 2003_CON_07_BC
20   gag.PEP. Fig. 79B. 2003_CON_07_BC gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 80A and 80B. Fig. 80A. 2003_CON_08_BC
gag.PEP. Fig. 80B. 2003_CON_08_BC gag.OPT.
(OPT = codon optimized encoding sequence.)

, Figures 81A and 81B. Fig. 81A. 2003_CON_10_CD
5   gag.PEP. Fig. 81B. 2003_CON_10_CD gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 82A and 82B. Fig. 82A.
2003_CON_11_CPX gag.PEP. Fig. 82B. 2003_CON_11_CPX
gag.OPT. (OPT = codon optimized encoding sequence.)

10   Figures 83A and 83B. Fig. 83A.
2003_CON_12_BF.gag.PEP. Fig. 83B.
2003_CON_12_BF.gag.OPT. (OPT = codon optimized
encoding sequence.)

Figures 84A and 84B. Fig. 84A. 2003_CON_14_BG
15   gag.PEP. Fig. 84B. 2003_CON_14_BG gag.OPT.
(OPT = codon optimized encoding sequence.)

Figures 85A and 85B. Fig. 85A. 2003_CONS
nef.PEP. Fig. 85B. 2003_CONS nef.OPT.
(OPT = codon optimized encoding sequence.)

20   Figures 86A and 86B. Fig. 86A. 2003_M
GROUP.anc nef.PEP. Fig. 86B. 2003_M
GROUP.anc.nef.OPT. (OPT = codon optimized encoding
sequence.)

Figures 87A and 87B. Fig. 87A. 2003_CON_A
nef.PEP. Fig. 87B. 2003_CON_A nef.OPT.
(OPT = codon optimized encoding sequence.)

Figures 88A-88D. Fig. 88A. 2003_CON_A1
5   nef.PEP. Fig. 88B. 2003_CON_A1 nef.OPT. Fig. 88C.
2003_A1.anc nef.PEP. Fig. 88D. 2003_A1.anc
nef.OPT. (OPT = codon optimized encoding sequence.)

Figures 89A and 89B. Fig. 89A. 2003_CON_A2
nef.PEP. Fig. 89B. 2003_CON_A2 nef.OPT.
10 (OPT = codon optimized encoding sequence.)

Figures 90A-90D. Fig. 90A. 2003_CON_B
nef.PEP. Fig. 90B. 2003_CON-B nef.OPT. Fig. 90C.
2003_B.anc nef.PEP. Fig. 90D. 2003_B.anc nef.OPT.
(OPT = codon optimized encoding sequence.)

15   Figures 91A and 91B. Fig. 91A. 2003_CON_02_AG
nef.PEP. Fig. 91B. 2003_CON_02_AG nef.OPT.
(OPT = codon optimized encoding sequence.)

Figures 92A-92D. Fig. 92A. 2003_CON_C
nef.PEP. Fig. 92B. 2003_CON_C nef.OPT. Fig. 92C.
20 2003_C.anc nef.PEP. Fig. 92D. 2003_C.anc nef.OPT.
(OPT = codon optimized encoding sequence.)

Figures 93A and 93B. Fig. 93A. 2003_CON_D
nef.PEP. Fig. 93B. 2003_CON_D nef.OPT.
(OPT = codon optimized encoding sequence.)

Figures 94A and 94B. Fig. 94A. 2003_CON_F1
5 nef.PEP. Fig. 94B. 2003_CON_F1 nef.OPT.
(OPT = codon optimized encoding sequence.)

Figures 95A and 95B. Fig. 95A. 2003_CON_F2
nef.PEP. Fig. 95B. 2003_CON_F2 nef.OPT.
(OPT = codon optimized encoding sequence.)

10   Figures 96A and 96B. Fig. 96A. 2003_CON_G
nef.PEP. Fig. 96B. 2003_CON_G nef.OPT.
(OPT = codon optimized encoding sequence.)

Figures 97A and 97B. Fig. 97A. 2003_CON_H
nef.PEP. Fig. 97B. 2003_CON_H nef.OPT.
15 (OPT = codon optimized encoding sequence.)

Figures 98A and 98B. Fig. 98A. 2003_CON_01_AE
nef.PEP. Fig. 98B. 2003_CON_01_AE nef.OPT.
(OPT = codon optimized encoding sequence.)

Figures 99A and 99B. Fig. 99A. 2003_CON_03_AE
20 nef.PEP. Fig. 99B. 2003_CON_03_AE nef.OPT.
(OPT = codon optimized encoding sequence.)

Figures 100A and 100B. Fig. 100A.
2003_CON_04_CFX nef.PEP. Fig. 100B.
2003_CON_04_CFX nef.OPT. (OPT = codon optimized
encoding sequence.)

5   Figures 101A and 101B. Fig. 101A.
2003_CON_06_CFX nef.PEP. Fig. 101B.
2003_CON_06_CFX nef.OPT. (OPT = codon optimized
encoding sequence.)

Figures 102A and 102B. Fig. 102A.
10 2003_CON_08_BC nef.PEP. Fig. 102B. 2003_CON_08_BC
nef.OPT. (OPT = codon optimized encoding sequence.)

Figures 103A and 103B. Fig. 103A.
2003_CON_10_CD nef.PEP. Fig. 103B. 2003_CON_10_CD
nef.OPT. (OPT = codon optimized encoding sequence.)

15   Figures 104A and 104B. Fig. 104A.
2003_CON_11_CFX nef.PEP. Fig. 104B.
2003_CON_11_CFX nef.OPT. (OPT = codon optimized
encoding sequence.)

Figures 105A and 105B. Fig. 105A.
20 2003_CON_12_BF nef.PEP. Fig. 105B. 2003_CON_12_BF
nef.OPT. (OPT = codon optimized encoding sequence.)

Figures 106A and 106B. Fig. 106A.
2003_CON_14_BG nef.PEP. Fig. 106B. 2003_CON_14_BG
nef.OPT. (OPT = codon optimized encoding sequence.)

Figures 107A and 107B. Fig. 107A. 2003_CON_S
5   pol.PEP. Fig. 107B. 2003_CON_S pol.OPT.
(OPT = codon optimized encoding sequence.)

Figures 108A and 108B. Fig. 108A. 2003_M
GROUP anc pol.PEP. Fig. 108B. 2003_M.GROUP anc
pol.OPT. (OPT = codon optimized encoding sequence.)

10   Figures 109A-109D. Fig. 109A. 2003_CON_A1
pol.PEP. Fig. 109B. 2003_CON_A1 pol.OPT.
Fig. 109C. 2003_A1.anc pol.PEP. Fig. 109D.
2003_A1.anc pol.OPT. (OPT = codon optimized
encoding sequence.)

15   Figures 110A and 110B. Fig. 110A. 2003_CON_A2
pol.PEP. Fig. 110B. 2003_CON_A2 pol.OPT.
(OPT = codon optimized encoding sequence.)

Figures 111A-111D. Fig. 111A. 2003_CON_B
pol.PEP. Fig. 111B. 2003_CON_B pol.OPT. Fig.
20   111C. 2003_B.anc pol.PEP. Fig. 111D. 2003_B.anc
pol.OPT. (OPT = codon optimized encoding sequence.)

Figures 112A-112D. Fig. 112A. 2003_CON_C
pol.PEP. Fig. 112B. 2003_CON_C pol.OPT.

Fig. 112C.   2003_C.anc pol.PEP.   Fig. 112D.
2003_C.anc pol.OPT.   (OPT = codon optimized encoding
sequence.)

5   Figures 113A and 113B.   Fig. 113A.   2003_CON_D
pol.PEP.   Fig. 113B.   2003_CON_D pol.OPT.
(OPT = codon optimized encoding sequence.)

Figures 114A and 114B.   Fig. 114A.   2003_CON_F1
pol.PEP.   Fig. 114B.   2003_CON_F1 pol.OPT.
(OPT = codon optimized encoding sequence.)

10   Figures 115A and 115B.   Fig. 115A.   2003_CON_F2
pol.PEP.   Fig. 115B.   2003_CON_F2 pol.OPT.
(OPT = codon optimized encoding sequence.)

Figures 116A and 116B.   Fig. 116A.   2003_CON_G
pol.PEP.   Fig. 116B.   2003_CON_G pol.OPT.
15   (OPT = codon optimized encoding sequence.)

Figures 117A and 117B.   Fig. 117A.   2003_CON_H
pol.PEP.   Fig. 117B.   2003_CON_H pol.OPT.
(OPT = codon optimized encoding sequence.)

Figures 118A and 118B.   Fig. 118A.
20   2003_CON_01_AE pol.PEP.   Fig. 118B.   2003_CON_01_AE
pol.OPT.   (OPT = codon optimized encoding sequence.)

Figures 119A and 119B.   Fig. 119A.
2003_CON_02_AG pol.PEP.   Fig. 119B.   2003_CON_02_AG
pol.OPT.   (OPT = codon optimized encoding sequence.)

Figures 120A and 120B.   Fig. 120A.

5   2003_CON_03_AB pol.PEP.   Fig. 120B.   2003_CON_03_AB
pol.OPT.   (OPT = codon optimized encoding sequence.)

Figures 121A and 121B.   Fig. 121A.
2003_CON_04_CPX pol.PEP.   Fig. 121B.
2003_CON_04_CPX pol.OPT.   (OPT = codon optimized

10   encoding sequence.)

Figures 122A and 122B.   Fig. 122A.
2003_CON_06_CPX pol.PEP.   Fig. 122B.
2003_CON_06_CPX pol.OPT.   (OPT = codon optimized
encoding sequence.)

15   Figures 123A and 123B.   Fig. 123A.
2003_CON_08_BC pol.PEP.   Fig. 123B.   2003_CON_08_BC
pol.OPT.   (OPT = codon optimized encoding sequence.)

Figures 124A and 124B.   Fig. 124A.
2003_CON_10_CD pol.PEP.   Fig. 124B.   2003_CON_10_CD

20   pol.OPT.   (OPT = codon optimized encoding sequence.)

Figures 125A and 125B.   Fig. 125A.
2003_CON_11_CPX pol.PEP.   Fig. 125B.

2003_CON_11_CPX pol.OPT.   (OPT = codon optimized
encoding sequence.)

Figures 126A and 126B.   Fig. 126A.
2003_CON_12_BF pol.PEP.   Fig. 126B.   2003_CON_12_BF

5   pol.OPT.   (OPT = codon optimized encoding sequence.)

Figures 127A and 127B.   Fig. 127A.
2003_CON_14_BG pol.PEP.   Fig. 127B.   2003_CON_14_BG
pol.OPT.   (OPT = codon optimized encoding sequence.)

DETAILED DESCRIPTION OF THE INVENTION

10   The present invention relates to an immunogen
that induces antibodies that neutralize a wide
spectrum of human immunodeficiency virus (HIV)
primary isolates and/or that induces a T cell
response.   The immunogen comprises at least one

15   consensus or ancestral immunogen (e.g., Env, Gag,
Nef or Pol), or portion or variant thereof.   The
invention also relates to nucleic acid sequences
encoding the consensus or ancestral immunogen, or
portion or variant thereof.   The invention further

20   relates to methods of using both the immunogen and
the encoding sequences.   While the invention is
described in detail with reference to specific
consensus and ancestral immunogens (for example, to
a group M consensus Env), it will be appreciated

25   that the approach described herein can be used to
generate a variety of consensus or ancestral

immunogens (for example, envelopes for other HIV-1
groups (e.g., N and O)).

       In accordance with one embodiment of the
invention, a consensus *env* gene can be constructed
5   by generating consensus sequences of *env* genes for
each subtype of a particular HIV-1 group (group M
being classified into subtypes A-D, F-H, J an K),
for example, from sequences in the Los Alamos HIV
Sequence Database (using, for example, MASE
10 (Multiple Aligned Sequence Editor)). A consensus
sequence of all subtype consensuses can then be
generated to avoid heavily sequenced subtypes
(Gaschen et al, Science 296:2354-2360 (2002), Korber
et al, Science 288:1789-1796 (2000)). In the case
15 of the group M consensus *env* gene described in
Example 1 (designated CON6), five highly variable
regions from a CRF08_BC recombinant strain (98CN006)
(V1, V2, V4, V5 and a region in cytoplasmic domain
of gp41) are used to fill in the missing regions in
20 the sequence (see, however, corresponding regions
for Con-S). For high levels of expression, the
codons of consensus or ancestral genes can be
optimized based on codon usage for highly expressed
human genes (Haas et al, Curr. Biol. 6:315-324
25 (2000), Andre et al, J. Virol. 72:1497-1503 (1998)).

       With the Year 1999 consensus group M *env* gene,
CON6, it has been possible to demonstrate induction
of superior T cell responses by CON6 versus wild-
type B and C *env* by the number of ELISPOT
30 γ-interferon spleen spot forming cells and the

number of epitopes recognized in two strains of mice
(Tables 1 and 2 show the data in BALB/c mice). The
ability of CON6 Env protein to induce neutralizing
antibodies to HIV-1 primary isolates has been
5 compared to that of several subtype B Env. The
target of neutralizing antibodies induced by CON6
includes several non-B HIV-1 strains.

Table 1. T cell epitope mapping of CON6, JRFL and 96ZM651
Env immunogen in BALB/c mice

Peptide	Immunogen			T cell response
	CON6	JRFL (B)	96ZM651 (C)	
CON 6 (group M consensus)				
16 DTEVHNVWATHACVP	+		+	CD4
48 KNSSEYYRLINCNTS / 49 EYYRLINCNTSAITQ	+		+	CD4
53 CPKVSFEPIPIHYCA / 54 SFEPIPIHYCAPAGF	+			CD4
62 NVSTVQCTHGIKPVV	+			CD4
104 ETITLPCRIKQIINM / 105 LPCRIKQIINMWQGV	+			CD8
130 GIVQQQSNLLRAIEA / 131 VQQSNLLRAIEAQQHL	+			CD4
134 AQQHLLQLTVWGIKQLQ / 135 LQLTVWGIKQLQARVL	+			CD4
Subtype B (MN)				
6223 AKAYDTEVHNVWATQ / 6224 DTEVHNVWATQACVP	+			CD4
6261 ACPKISFEPIPIHYC / 6262 ISFEPIPIHYCAPAG	+			CD4
6286 RKRIHIGPGRAFYTT / 6287 HIGPGRAFYTTKNII		+		CD8
6346 IVQQQNNLLRAIEAQ / 6347 QNNLLRAIEAQQHML	+			CD4
Subtype C (Chn19)				
4834 VPVWKEAKTTLFCASDAKSY			+	CD4
4838 GKEVHNVWATHACVPTDPNP	+		+	CD4
4848 SSENSSEYYRLINCNTSAIT	+		+	CD4
4854 STVQCTHGIKPVVSTQLLLN	+			CD4
4884 QQSNLLRAIEAQQHLLQLTV	+			CD4
4885 AQQHLLQLTVWGIKQLQTRV	+			CD4

Table 2.   T cell epitope mapping of CON6.gp120
             immunogen in C57BL/6 mice

Peptide	Peptide sequence	T cell response
CON 6 (consensus)		
2	GIQRNCQHLWRWGTM	CD8
3	NCQHLWRWGTMILGM	
16	DTEVHNVWATHACVP	CD4
53	CPKVSFEPIPIHYCA	CD4
97	FYCNTSGLFNSTWMF	CD8
99	FNSTWMFNGTYMFNG	CD8
Subtype B (MN)		
6210	GIRRNYQHWWGWGTM	CD8
6211	NYQHWWGWGTMLLGL	
6232	NMWKNNMVEQMHEDI	CD4
6262	ISFEPIPIHYCAPAG	CD4
6290	NIIGTIRQAHCNISR	CD4
6291	TIRQAHCNISRAKWN	
Subtype C (Chn 19)		
4830	MRVTGIRKNYQHLWRWGTML	CD8
5446	RWGTMLLGMLMICSAAEN	CD8
4836	GKEVHNVWATHACVPTDPNP	CD4
4862	GDIRQAHCNISKDKWNETLQ	CD4
4888	LLGIWGCSGKLICTTTVPWN	CD8

For the Year 2000 consensus group M *env* gene,

5   Con-S, the Con-S envelope has been shown to be as

immunogenic as the CON6 envelope gene in T cell γ

interferon ELISPOT assays in two strains of mice

(the data for C57BL/6 are shown in Fig. 27).
Furthermore, in comparing CON6 and Con-S gp140 Envs
as protein immunogens for antibody in guinea pigs
(Table 3), both gp140 Envs were found to induce
antibodies that neutralized subtype B primary
isolates. However, Con-S gp140 also induced robust
neutralization of the subtype C isolates TV-1 and DU
123 as well as one subtype A HIV-1 primary isolate,
while CON6 did not.

TABLE 3   Ability of Group M Consensus CON6 and Con-S Envs to Induce Neutralization of HIV-1 Primary Isolates

HIV-1 Isolate (Subtype)	CON6 gp140CF				CON6 gp140 CFI				CONS gp140 CFI			
	770	771	772	775	781	783	784	786	776	777	778	780
BX08(B)	520	257	428	189	218	164	>540	199	>540	>540	>540	>540
QH0692 (B)	46	55	58	77	<20	91	100	76	109	<20	<20	<20
SS1196(B)	398	306	284	222	431	242	>540	351	>540	296	>540	>540
JRLFL(B)	<20	<20	<20	<20	<20	169	<20	<20	<20	<20	<20	<20
BG1168(B)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
3988(B)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
6101(B)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
TV-1(C)	<20	<20	<20	<20	<20	<20	<20	<20	356	439	>540	>54
DU123(C)	<20	<20	71	74	<20	72	<20	<20	176	329	387	378
DU172(C)	<20	<20	96	64	<20	<20	<20	<20	<20	235	<20	213
ZM18108.6(C)	ND	ND	ND	ND	<20	<20	<20	<20	84	61	86	43

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ZM14654.7(C)	ND	ND	ND	<20	<20	<20	<20	<20	<20	<20	30	<20
DU151(C)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
DU422(C)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
DU156(C)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
92RWO20(A)	<20	<20	<20	<20	<20	44	<20	<20	116	204	95	177
92UG037(A)	<20	<20	30	<20	<20	<20	<20	<20	<20	<20	<20	<20

‡ 50% Neutralization titers after 4th or 5th immunizations

Year 2000 Con-S 140CFI.ENV sequence is shown in Fig. 26A. Gp140 CFI refers to an HIV-1 envelope design in which the cleavage-site is deleted (c), the fusion-site is deleted (F) and the gp41 immunodominant region is deleted (I), in addition to the deletion of transmembrane and cytoplasmic domains. The codon-optimized Year 2000 Con-S 140 CFI sequence is shown in Fig. 26B.

As the next iteration of consensus immunogens, and in recognition of the fact that a practical HIV-1 immunogen can be a polyvalent mixture of either several subtype consensus genes, a mixture of subtype and consensus genes, or a mixture of centralized genes and wild type genes, a series of 11 subtype consensus, and wild type genes have been designed from subtypes A, B, C, CRF AE01, and G as well as a group M consensus gene from Year 2003 Los Alamos National Database sequences. The wild type sequences were chosen either because they were known to come from early transmitted HIV-1 strains (those strains most likely to be necessary to be protected against by a vaccine) or because they were the most recently submitted strains in the database of that subtype. These nucleotide and amino acid sequences are shown in Figures 28-38 (for all 140CF designs shown, 140CF gene can be flanked with the 5' sequence "TTCAGTCGACGGCCACC" that contains a Kozak sequence (GCCACCATGG/A) and *Sal*I site and 3' sequence of TAAAGATCTTACAA containing stop codon and *Bgl*II site). Shown in Figures 39-62 are 2003 centralized (consensus and ancestral) HIV-1 envelope proteins and the codon optimized gene sequences.

Major differences between CON6 gp140 (which does not neutralize non-clade B HIV strains) and Con-S gp140 (which does induce antibodies that neutralize non-clade B HIV strains) are in Con-S V1, V2, V4 and V5 regions. For clade B strains, peptides of the V3 region can induce neutralizing

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antibodies (Haynes et al, J. Immunol. 151:1646-1653
(1993)). Thus, construction of Th-V1, Th-V2, Th-
V4, Th-V5 peptides can be expected to give rise to
the desired broadly reactive anti-non-clade B

5   neutralizing antibodies. Therefore, the Th-V
peptides set forth in Table 4 are contemplated for
use as a peptide immunogen(s) derived from Con-S
gp140. The gag Th determinant (GTH, Table 4) or any
homologous GTH sequence in other HIV strains, can be

10 used to promote immunogenicity and the C4 region of
HIV gp120 can be used as well (KQIINMWQVVGKAMYA) or
any homologous C4 sequence from other HIV strains
(Haynes et al, J. Immunol. 151:1646-1653 (1993)).
Con-S V1, V2, V4, V5 peptides with an N-terminal

15 helper determinant can be used singly or together,
when formulated in a suitable adjuvant such as
Corixa's RC529 (Baldridge et al, J. Endotoxin Res.
8:453-458 (2002)), to induce broadly cross reactive
neutralizing antibodies to non-clade B isolates.

20

Table 4		
1)	GTH Con-S V1 132-150	YKRWIILGLNKIVRMYTNVNVTNTTNNTEEKGEIKN
2)	GTH Con-S V2 157-189	YKRWIILGLNKIVRMYTEIRDKKQKVYALFYRLDVVPIDDNNNNSSNYR
3)	GTH Con-S V3 294-315	YKRWIILGLNKIVRMYTRPNNNTRKSIRIGPGQAFYAT
4)	GTH Con-S V4 381-408	YKRWIILGLNKIVRMYNTSGLFNSTWIGNGTKNNNNTNDTITLP
5)	GTH Con-S V5 447-466	YKRWIILGLNKIVRMYRDGGNNNTNETEIFRPGGGD
6)	GTH Con-6 V1 132-150	YKRWIILGLNKIVRMYNVRNVSSNGTETDNEEIKN
7)	GTH Con-6 V2 157-196	YKRWIILGLNKIVRMYTELRDKKQKVYALFYRLDVVPIDDKNSSEISGKNSSEYYR
8)	GTH-Con6 V3 301-322	YKRWIILGLNKIVRMYTRPNNNTRKSIHIGPGQAFYAT
9)	GTH Con-6 V4 388-418	YKRWIILGLNKIVRMYNTSGLFNSTWMFNGTYMFNGTKDNSETITLP
10	GTH Con 6 V5 457-477	YKRWIILGLNKIVRMYRDGGNNSNKNKTETFRPGGGD

It will be appreciated that the invention
includes portions and variants of the sequences
specifically disclosed herein. For example, forms
5   of codon optimized consensus encoding sequences can
be constructed as gp140CF, gp140 CFI, gp120 or gp160
forms with either gp120/41 cleaved or uncleaved.
For example, and as regards the consensus and
ancestral envelope sequences, the invention
10   encompasses envelope sequences devoid of V3.
Alternatively, V3 sequences can be selected from
preferred sequences, for example, those described in
U.S. Application No. 10/431,596 and U.S. Provisional
Application No. 60/471,327. In addition, an optimal
15   immunogen for breadth of response can include
mixtures of group M consensus *gag*, *pol*, *nef* and *env*
encoding sequences, and as well as consist of

mixtures of subtype consensus or ancestral encoding
sequences for *gag, pol, nef* and *env* HIV genes. For
dealing with regional differences in virus strains,
an efficacious mixture can include mixtures of

5   consensus/ancestral and wild type encoding
sequences.

A consensus or ancestral envelope of the
invention can be been "activated" to expose
intermediate conformations of neutralization

10   epitopes that normally are only transiently or less
well exposed on the surface of the HIV virion. The
immunogen can be a "frozen" triggered form of a
consensus or ancestral envelope that makes available
specific epitopes for presentation to B lymphocytes.

15   The result of this epitope presentation is the
production of antibodies that broadly neutralize
HIV. (Attention is directed to WO 02/024149 and to
the activated/triggered envelopes described
therein.)

20   The concept of a fusion intermediate immunogen
is consistent with observations that the gp41 HR-2
region peptide, DP178, can capture an uncoiled
conformation of gp41 (Furata et al, Nature Struct.
Biol. 5:276 (1998)), and that formalin-fixed HIV-

25   infected cells can generate broadly neutralizing
antibodies (LaCasse et al, Science 283:357 (1997)).
Recently a monoclonal antibody against the coiled-
coil region bound to a conformational determinant of
gp41 in HR1 and HR2 regions of the coiled-coil gp41

30   structure, but did not neutralize HIV (Jiang et al,
J. Virol. 10213 (1998)). However, this latter study

proved that the coiled-coil region is available for
antibody to bind if the correct antibody is
generated.

5   The immunogen of one aspect of the invention
comprises a consensus or ancestral envelope either
in soluble form or anchored, for example, in cell
vesicles or in liposomes containing translipid
bilayer envelope. To make a more native envelope,
gp140 or gp160 consensus or ancestral sequences can
10   be configured in lipid bilayers for native trimeric
envelope formation. Alternatively, triggered gp160
in aldrithio 1-2 inactivated HIV-1 virions can be
used as an immunogen. The gp160 can also exist as a
recombinant protein either as gp160 or gp140 (gp140
15   is gp160 with the transmembrane region and possibly
other gp41 regions deleted). Bound to gp160 or
gp140 can be recombinant CCR5 or CXCR4 co-receptor
proteins (or their extracellular domain peptide or
protein fragments) or antibodies or other ligands
20   that bind to the CXCR4 or CCR5 binding site on
gp120, and/or soluble CD4, or antibodies or other
ligands that mimic the binding actions of CD4.
Alternatively, vesicles or liposomes containing CD4,
CCR5 (or CXCR4), or soluble CD4 and peptides
25   reflective of CCR5 or CXCR4 gp120 binding sites.
Alternatively, an optimal CCR5 peptide ligand can be
a peptide from the N-terminus of CCR5 wherein
specific tyrosines are sulfated (Bormier et al,
Proc. Natl. Acad. Sci. USA 97:5762 (2001)). The
30   triggered immunogen may not need to be bound to a
membrane but may exist and be triggered in solution.

Alternatively, soluble CD4 (sCD4) can be replaced by
an envelope (gp140 or gp160) triggered by CD4
peptide mimetopes (Vitra et al, Proc. Natl. Acad.
Sci. USA 96:1301 (1999)). Other HIV co-receptor

5   molecules that "trigger" the gp160 or gp140 to
undergo changes associated with a structure of gp160
that induces cell fusion can also be used. Ligation
of soluble HIV gp140 primary isolate HIV 89.6
envelope with soluble CD4 (sCD4) induced

10   conformational changes in gp41.

        In one embodiment, the invention relates to an
immunogen that has the characteristics of a receptor
(CD4)-ligated consensus or ancestral envelope with
CCR5 binding region exposed but unlike CD4-ligated

15   proteins that have the CD4 binding site blocked,
this immunogen has the CD4 binding site exposed
(open). Moreover, this immunogen can be devoid of
host CD4, which avoids the production of potentially
harmful anti-CD4 antibodies upon administration to a

20   host.

        The immunogen can comprise consensus or
ancestral envelope ligated with a ligand that binds
to a site on gp120 recognized by an A32 monoclonal
antibodies (mab) (Wyatt et al, J. Virol. 69:5723

25   (1995), Boots et al, AIDS Res. Hum. Retro. 13:1549
(1997), Moore et al, J. Virol. 68:8350 (1994),
Sullivan et al, J. Virol. 72:4694 (1998), Fouts et
al, J. Virol. 71:2779 (1997), Ye et al, J. Virol.
74:11955 (2000)). One A32 mab has been shown to

30   mimic CD4 and when bound to gp120, upregulates
(exposes) the CCR5 binding site (Wyatt et al, J.

Virol. 69:5723 (1995)). Ligation of gp120 with such
a ligand also upregulates the CD4 binding site and
does not block CD4 binding to gp120.
Advantageously, such ligands also upregulate the HR-
2 binding site of gp41 bound to cleaved gp120,
uncleaved gp140 and cleaved gp41, thereby further
exposing HR-2 binding sites on these proteins - each
of which are potential targets for anti-HIV
neutralizing antibodies.

        In a specific aspect of this embodiment, the
immunogen comprises soluble HIV consensus or
ancestral gp120 envelope ligated with either an
intact A32 mab, a Fab2 fragment of an A32 mab, or a
Fab fragment of an A32 mab, with the result that the
CD4 binding site, the CCR5 binding site and the HR-2
binding site on the consensus or ancestral envelope
are exposed/upregulated. The immunogen can comprise
consensus or ancestral envelope with an A32 mab (or
fragment thereof) bound or can comprise consensus or
ancestral envelope with an A32 mab (or fragment
thereof) bound and cross-linked with a cross-linker
such as .3% formaldehyde or a heterobifunctional
cross-linker such as DTSSP (Pierce Chemical
Company). The immunogen can also comprise uncleaved
consensus or ancestral gp140 or a mixture of
uncleaved gp140, cleaved gp41 and cleaved gp120. An
A32 mab (or fragment thereof) bound to consensus or
ancestral gp140 and/or gp120 or to gp120 non-
covalently bound to gp41, results in upregulation
(exposure) of HR-2 binding sites in gp41, gp120 and
uncleaved gp140. Binding of an A32 mab (or fragment

thereof) to gp120 or gp140 also results in
upregulation of the CD4 binding site and the CCR5
binding site. As with gp120 containing complexes,
complexes comprising uncleaved gp140 and an A32 mab

5   (or fragment thereof) can be used as an immunogen
uncross-linked or cross-linked with cross-linker
such as .3% formaldehyde or DTSSP. In one
embodiment, the invention relates to an immunogen
comprising soluble uncleaved consensus or ancestral

10   gp140 bound and cross linked to a Fab fragment or
whole A32 mab, optionally bound and cross-linked to
an HR-2 binding protein.

        The consensus or ancestral envelope protein
triggered with a ligand that binds to the A32 mab

15   binding site on gp120 can be administered in
combination with at least a second immunogen
comprising a second envelope, triggered by a ligand
that binds to a site distinct from the A32 mab
binding site, such as the CCR5 binding site

20   recognized by mab 17b. The 17b mab (Kwong et al,
Nature 393:648 (1998) available from the AIDS
Reference Repository, NIAID, NIH) augments sCD4
binding to gp120. This second immunogen (which can
also be used alone or in combination with triggered

25   immunogens other than that described above) can, for
example, comprise soluble HIV consensus or ancestral
envelope ligated with either the whole 17b mab, a
Fab2 fragment of the 17b mab, or a Fab fragment of
the 17b mab. It will be appreciated that other CCR5

30   ligands, including other antibodies (or fragments
thereof), that result in the CD4 binding site being

exposed can be used in lieu of the 17b mab. This
further immunogen can comprise gp120 with the 17b
mab, or fragment thereof, (or other CCR5 ligand as
indicated above) bound or can comprise gp120 with

5   the 17b mab, or fragment thereof, (or other CCR5
ligand as indicated above) bound and cross-linked
with an agent such as .3% formaldehyde or a
heterobifunctional cross-linker, such as DTSSP
(Pierce Chemical Company). Alternatively, this

10   further immunogen can comprise uncleaved gp140
present alone or in a mixture of cleaved gp41 and
cleaved gp120. Mab 17b, or fragment thereof (or
other CCR5 ligand as indicated above) bound to gp140
and/or gp120 in such a mixture results in exposure

15   of the CD4 binding region. The 17b mab, or fragment
thereof, (or other CCR5 ligand as indicated above)
gp140 complexes can be present uncross-linked or
cross-linked with an agent such as .3% formaldehyde
or DTSSP.

20   Soluble HR-2 peptides, such as T649Q26L and
DP178, can be added to the above-described complexes
to stabilize epitopes on consensus gp120 and gp41 as
well as uncleaved consensus gp140 molecules, and can
be administered either cross-linked or uncross-

25   linked with the complex.

         A series of monoclonal antibodies (mabs) have
been made that neutralize many HIV primary isolates,
including, in addition to the 17b mab described
above, mab IgG1b12 that binds to the CD4 binding

30   site on gp120(Roben et al, J. Virol. 68:482 (1994),
Mo et al, J. Virol. 71:6869 (1997)), mab 2G12 that

binds to a conformational determinant on gp120
(Trkola et al, J. Virol. 70:1100 (1996)), and mab
2F5 that binds to a membrane proximal region of gp41
(Muster et al, J. Virol. 68:4031 (1994)).

5   As indicated above, various approaches can be
used to "freeze" fusogenic epitopes in accordance
with the invention. For example, "freezing" can be
effected by addition of the DP-178 or T-649Q26L
peptides that represent portions of the coiled coil
10   region, and that when added to CD4-triggered
consensus or ancestral envelope, result in
prevention of fusion (Rimsky et al, J. Virol.
72:986-993 (1998)). HR-2 peptide bound consensus or
ancestral gp120, gp140, gp41 or gp160 can be used as
15   an immunogen or crosslinked by a reagent such as
DTSSP or DSP (Pierce Co.), formaldehyde or other
crosslinking agent that has a similar effect.

        "Freezing" can also be effected by the addition
of 0.1% to 3% formaldehyde or paraformaldehyde, both
20   protein cross-linking agents, to the complex, to
stabilize the CD4, CCR5 or CXCR4, HR-2 peptide gp160
complex, or to stabilize the "triggered" gp41
molecule, or both (LaCasse et al, Science 283:357-
362 (1999)).

25   Further, "freezing" of consensus or ancestral
gp41 or gp120 fusion intermediates can be effected
by addition of heterobifunctional agents such as DSP
(dithiobis[succimidylproprionate]) (Pierce Co.
Rockford, ILL., No. 22585ZZ) or the water soluble
30   DTSSP (Pierce Co.) that use two NHS esters that are
reactive with amino groups to cross link and

stabilize the CD4, CCR5 or CXCR4, HR-2 peptide gp160
complex, or to stabilize the "triggered" gp41
molecule, or both.

5   Analysis of T cell immune responses in
immunized or vaccinated animals and humans shows
that the envelope protein is normally not a main
target for T cell immune response although it is the
only gene that induces neutralizing antibodies.
HIV-1 Gag, Pol and Nef proteins induce a potent T
10   cell immune response. Accordingly, the invention
includes a repertoire of consensus or ancestral
immunogens that can induce both humoral and cellular
immune responses. Subunits of consensus or
ancestral sequences can be used as T or B cell
15   immunogens. (See Examples 6 and 7, and Figures
referenced therein, and Figures 63-127.

The immunogen of the invention can be
formulated with a pharmaceutically acceptable
carrier and/or adjuvant (such as alum) using
20   techniques well known in the art. Suitable routes
of administration of the present immunogen include
systemic (e.g. intramuscular or subcutaneous).
Alternative routes can be used when an immune
response is sought in a mucosal immune system (e.g.,
25   intranasal).

The immunogens of the invention can be
chemically synthesized and purified using methods
which are well known to the ordinarily skilled
artisan. The immunogens can also be synthesized by
30   well-known recombinant DNA techniques. Nucleic
acids encoding the immunogens of the invention can

be used as components of, for example, a DNA vaccine
wherein the encoding sequence is administered as
naked DNA or, for example, a minigene encoding the
immunogen can be present in a viral vector. The

5   encoding sequence can be present, for example, in a
replicating or non-replicating adenoviral vector, an
adeno-associated virus vector, an attenuated
mycobacterium tuberculosis vector, a Bacillus
Calmette Guerin (BCG) vector, a vaccinia or Modified

10   Vaccinia Ankara (MVA) vector, another pox virus
vector, recombinant polio and other enteric virus
vector, Salmonella species bacterial vector,
Shigella species bacterial vector, Venezuelean
Equine Encephalitis Virus (VEE) vector, a Semliki

15   Forest Virus vector, or a Tobacco Mosaic Virus
vector. The encoding sequence, can also be
expressed as a DNA plasmid with, for example, an
active promoter such as a CMV promoter. Other live
vectors can also be used to express the sequences of

20   the invention. Expression of the immunogen of the
invention can be induced in a patient's own cells,
by introduction into those cells of nucleic acids
that encode the immunogen, preferably using codons
and promoters that optimize expression in human

25   cells. Examples of methods of making and using DNA
vaccines are disclosed in U.S. Pat. Nos. 5,580,859,
5,589,466, and 5,703,055.

The composition of the invention comprises an
immunologically effective amount of the immunogen of

30   this invention, or nucleic acid sequence encoding
same, in a pharmaceutically acceptable delivery

system.   The compositions can be used for prevention
and/or treatment of immunodeficiency virus
infection.   The compositions of the invention can be
formulated using adjuvants, emulsifiers,

5   pharmaceutically-acceptable carriers or other
ingredients routinely provided in vaccine
compositions.   Optimum formulations can be readily
designed by one of ordinary skill in the art and can
include formulations for immediate release and/or

10   for sustained release, and for induction of systemic
immunity and/or induction of localized mucosal
immunity (e.g, the formulation can be designed for
intranasal administration).   The present
compositions can be administered by any convenient

15   route including subcutaneous, intranasal, oral,
intramuscular, or other parenteral or enteral route.
The immunogens can be administered as a single dose
or multiple doses.   Optimum immunization schedules
can be readily determined by the ordinarily skilled

20   artisan and can vary with the patient, the
composition and the effect sought.

          The invention contemplates the direct use of
both the immunogen of the invention and/or nucleic
acids encoding same and/or the immunogen expressed

25   as minigenes in the vectors indicated above.   For
example, a minigene encoding the immunogen can be
used as a prime and/or boost.

          The invention includes any and all amino acid
sequences disclosed herein and, where applicable, CF

30   and CFI forms thereof, as well as nucleic acid

sequences encoding same (and nucleic acids
complementary to such encoding sequences).

Certain aspects of the invention can be
described in greater detail in the non-limiting
5   Examples that follows.

EXAMPLE 1

Artificial HIV-1 Group M Consensus Envelope

EXPERIMENTAL DETAILS

10   *Expression of CON6 gp120 and gp140 proteins in
recombinant vaccinia viruses (VV).* To express and
purify the secreted form of HIV-1 CON6 envelope
proteins, CON6 gp120 and gp140CF plasmids were
constructed by introducing stop codons after the
15   gp120 cleavage site (REKR) and before the
transmembrane domain (YIKIFIMIVGGLIGLRIVFAVLSIVN),
respectively. The gp120/gp41 cleavage site and
fusion domain of gp41 were deleted in the gp140CF
protein. Both CON6 gp120 and gp140CF DNA constructs
20   were cloned into the pSC65 vector (from Bernard
Moss, NIH, Bethesda, MD) at SalI and KpnI
restriction enzyme sites. This vector contains the
lacZ gene that is controlled by the p7.5 promoter.
A back-to-back P E/L promoter was used to express
25   CON6 *env* genes. BSC-1 cells were seeded at 2×10^5
in each well in a 6-well plate, infected with wild-
type vaccinia virus (WR) at a MOI of 0.1 pfu/cell,
and 2 hr after infection, pSC65-derived plasmids

containing CON6 *env* genes were transfected into the
VV-infected cells and recombinant (r) VV selected as
described (Moss and Earl, Current Protocols in
Molecular Biology, eds, Ausubel et al (John Wiley &
5   Sons, Inc. Indianapolis, IN) pp. 16.15.1-16.19.9
(1998)). Recombinant VV that contained the CON6 *env*
genes were confirmed by PCR and sequencing analysis.
Expression of the CON6 envelope proteins was
confirmed by SDS-PAGE and Western blot assay.
10 Recombinant CON6 gp120 and gp140CF were purified
with agarose *galanthus Nivalis* lectin beads (Vector
Labs, Burlingame, CA), and stored at -70°C until use.
Recombinant VV expressing JRFL (vCB-28) or 96ZM651
(vT241R) gp160 were obtained from the NIH AIDS
15 Research and Reference Reagent Program (Bethesda,
MD).

     Monoclonal Antibodies and gp120 Wild-type
     Envelopes. Human mabs against a conformational
20 determinant on gp120 (A32), the gp120 V3 loop (F39F)
and the CCR5 binding site (17b) were the gifts of
James Robinson (Tulane Medical School, New Orleans,
LA) (Wyatt et al, Nature 393;705-711 (1998), Wyatt
et al, J. Virol. 69:5723-5733 (1995)). Mabs 2F5,
25 447, b12, 2G12 and soluable CD4 were obtained from
the NIH AIDS Research and Reference Reagent Program
(Bethesda, MD) (Gorny et al, J. Immunol. 159:5114-
5122 (1997), Nyambi et al, J. Virol. 70:6235-6243
(1996), Purtscher et al, AIDS Res. Hum. Retroviruses
30 10:1651-1658 (1994), Trkola et al, J. Virol 70:1100-
1108 (1996)). T8 is a murine mab that maps to the

gp120 C1 region (a gift from P. Earl, NIH, Bethesda,
MD). BaL (subtype B), 96ZM651 (subtype C), and
93TH975 (subtype E) gp120s were provided by QBI,
Inc. and the Division of AIDS, NIH. CHO cell lines
5   that express 92U037 (subtype A) and 93BR029 (subtype
F) gp140 (secreted and uncleaved) were obtained from
NICBS, England.

   Surface Plasmon Resonance Biosensor (SPR)
10   *Measurements and ELISA.* SPR biosensor measurements
were determined on a BIAcore 3000 instrument
(BIAcore Inc., Uppsala, Sweden) instrument and data
analysis was performed using BIAevaluation 3.0
software (BIAcore Inc, Upsaala, Sweden). Anti-gp120
15   mabs (T8, A32, 17b, 2G12) or sCD4 in 10mM Na-acetate
buffer, pH 4.5 were directly immobilized to a CM5
sensor chip using a standard amine coupling protocol
for protein immobilization. FPLC purified CON6
gp120 monomer or gp140CF oligomer recombinant
20   proteins were flowed over CM5 sensor chips at
concentrations of 100 and 300 μg/ml, respectively.
A blank in-line reference surface (activated and de-
activated for amine coupling) or non-bonding mab
controls were used to subtract non-specific or bulk
25   responses. Soluble 89.6 gp120 and irrelevant IgG
was used as a positive and negative control
respectively and to ensure activity of each mab
surface prior to injecting the CON6 Env proteins.
Binding of CON6 envelope proteins was monitored in
30   real-time at 25°C with a continuous flow of PBS (150
mM NaCl, 0.005% surfactant P20), pH 7.4 at 10-30

μl/min. Bound proteins were removed and the sensor
surfaces were regenerated following each cycle of
binding by single or duplicate 5-10 μl pulses of
regeneration solution (10 mM glycine-HCl, pH 2.9).

5   ELISA was performed to determine the reactivity of
various mabs to CON6 gp120 and gp140CF proteins as
described (Haynes et al, AIDS Res. Hum. Retroviruses
11:211-221 (1995)). For assay of human mab binding
to rgp120 or gp140 proteins, end-point titers were

10   defined as the highest titer of mab (beginning at 20
μg/ml) at which the mab bound CON6 gp120 and gp140CF
Env proteins ≥ 3 fold over background control (non-
binding human mab).

15   *Infectivity and coreceptor usage assays.* HIV-
1/SG3Δenv and CON6 or control *env* plasmids were
cotransfected into human 293T cells. Pseudotyped
viruses were harvested, filtered and p24
concentration was quantitated (DuPont/NEN Life

20   Sciences, Boston, MA). Equal amounts of p24 (5 ng)
for each pseudovirion were used to infect JC53-BL
cells to determine the infectivity (Derdeyn e al, J.
Virol. 74:8358-8367 (2000), Wei et al, Antimicrob
Agents Chemother. 46:1896-1905 (2002)). JC53-BL

25   cells express CD4, CCR5 and CXCR4 receptors and
contain a β-galactosidase (β-gal) gene stably
integrated under the transcriptional control of an
HIV-1 long terminal repeat (LTR). These cells can
be used to quantify the infectious titers of

30   pseudovirion stocks by staining for β-gal expression

and counting the number of blue cells (infectious
units) per microgram of p24 of pseudovirons (IU/μg
p24) (Derdeyn e al, J. Virol. 74:8358-8367 (2000),
Wei et al, Antimicrob Agents Chemother. 46:1896-1905
5   (2002)). To determine the coreceptor usage of the
CON6 *env* gene, JC53BL cells were treated with 1.2 μM
AMD3100 and 4 μM TAK-799 for 1 hr at 37°C then
infected with equal amounts of p24 (5 ng) of each
Env pseudotyped virus. The blockage efficiency was
10   expressed as the percentage of the infectious units
from blockage experiments compared to that from
control culture without blocking agents. The
infectivity from control group (no blocking agent)
was arbitrarily set as 100%.

15

Immunizations. All animals were housed in the
Duke University Animal Facility under AALAC
guidelines with animal use protocols approved by the
Duke University Animal Use and Care Committee.
20   Recombinant CON6 gp120 and gp140CF glycoproteins
were formulated in a stable emulsion with RIBI-CWS
adjuvant based on the protocol provided by the
manufacturer (Sigma Chemical Co., St. Louis, MO).
For induction of anti-envelope antibodies, each of
25   four out-bred guinea pigs (Harlan Sprague, Inc.,
Chicago, IL) was given 100 μg either purified CON6
gp120 or gp140CF subcutaneously every 3 weeks (total
of 5 immunizations). Serum samples were heat-
inactivated (56°C, 1 hr), and stored at -20°C until
30   use.

For induction of anti-envelope T cell
responses, 6-8 wk old female BALB/c mice (Frederick
Cancer Research and Developmental Center, NCI,
Frederick, MD) were immunized i.m. in the quadriceps
5   with 50 μg plasmid DNA three times at a 3-week
interval. Three weeks after the last DNA
immunization, mice were boosted with 10^7 PFU of rVV
expressing Env proteins. Two weeks after the boost,
all mice were euthanized and spleens were removed
10 for isolation of splenocytes.

Neutralization assays. Neutralization assays
were performed using either a MT-2 assay as
described in Bures et al, AIDS Res. Hum.
15 Retroviruses 16:2019-2035 (2000), a luciferase-based
multiple replication cycle HIV-1 infectivity assay
in 5.25.GFP.Luc.M7 cells using a panel of HIV-1
primary isolates (Bures et al, AIDS Res. Hum.
Retroviruses 16:2019-2035 (2000), Bures et al, J.
20 Virol. 76:2233-2244 (2002)), or a syncytium (fusion
from without) inhibition assay using inactivated
HIV-1 virions (Rossio et al, J. Virol. 72:7992-8001
(1998)). In the luciferase-based assay,
neutralizing antibodies were measured as a function
25 of a reduction in luciferase acitivity in
5.25.EGFP.Luc.M7 cells provided by Nathaniel R.
Landau, Salk Institute, La Jolla, CA (Brandt et al,
J. Biol. Chem. 277:17291-17299 (2002)). Five
hundred tissue culture infectious dose 50 ($TCID_{50}$) of
30 cell-free virus was incubated with indicated serum

dilutions in 150 µl (1 hr, at 37°C) in triplicate in 96-well flat-bottom culture plates. The 5.25.EGFP.Luc.M7 cells were suspended at a density of 5 x 10^5/ml in media containing DEAE dextran (10

5   µg/ml). Cells (100 µl) were added and until 10% of cells in control wells (no test serum sample) were positive for GFP expression by fluorescence microscopy. At this time the cells were concentrated 2-fold by removing one-half volume of

10   media. A 50 µl suspension of cells was transferred to 96-well white solid plates (Costar, Cambridge, MA) for measurement of luciferase activity using Bright-Glo™ substrate (Promega, Madison, WI) on a Wallac 1420 Multilabel Counter (PerkinElmer Life

15   Sciences, Boston, MA). Neutralization titers in the MT-2 and luciferase assays were those where $\geq$ 50% virus infection was inhibited. Only values that titered beyond 1:20 (i.e. >1:30) were considered significantly positive. The syncytium inhibition

20   "fusion from without" assay utilized HIV-1 aldrithiol-2 (AT-2) inactivated virions from HIV-1 subtype B strains ADA and AD8 (the gift of Larry Arthur and Jeffrey Lifson, Frederick Research Cancer Facility, Frederick, MD) added to SupT1 cells, with

25   syncytium inhibition titers determined as those titers where $\geq$90% of syncytia were inhibited compared to prebleed sera.

Enzyme linked immune spot (ELISPOT) assay.

30   Single-cell suspensions of splenocytes from

individual immunized mice were prepared by mincing
and forcing through a 70 μm Nylon cell strainer (BD
Labware, Franklin Lakes, NJ). Overlapping Env
peptides of CON6 gp140 (159 peptides, 15mers
5   overlapping by 11) were purchased from Boston
Bioscence, Inc (Royal Oak, MI). Overlapping Env
peptides of MN gp140 (subtype B; 170 peptides,
15mers overlapping by 11) and Chn19 gp140 (subtype
C; 69 peptides, 20mers overlapping by 10) were
10   obtained from the NIH AIDS Research and Reference
Reagent Program (Bethesda, MD). Splenocytes (5
mice/group) from each mouse were stimulated *in vitro*
with overlapping Env peptides pools from CON6,
subtype B and subtype C Env proteins. 96-well PVDF
15   plates (MultiScreen-IP, Millipore, Billerica, MA)
were coated with anti-IFN-γ mab (5 μg/ml, AN18;
Mabtech, Stockholm, Sweden). After the plates were
blocked at 37°C for 2 hr using complete Hepes
buffered RPMI medium, 50μl of the pooled overlapping
20   envelope peptides (13 CON6 and MN pools, 13-14
peptides in each pool; 9 Chn19 pool, 7-8 peptide in
each pool) at a final concentration of 5 μg/ml of
each were added to the plate. Then 50 μl of
splenocytes at a concentration of 1.0 X 10^7/ml were
25   added to the wells in duplicate and incubated for 16
hr at 37°C with 5% CO_2. The plates were incubated
with 100 μl of a 1:1000 dilution of streptavidin
alkaline phosphatase (Mabtech, Stockholm, Sweden),
and purple spots developed using 100 μl of BCIP/NBT
30   (Plus) Alkaline Phosphatase Substrate (Moss,

Pasadena, MD). Spot forming cells (SFC) were
measured using an Immunospot counting system (CTL
Analyzers, Cleveland, OH). Total responses for each
envelope peptide pool are expressed as SFCs per 10^6

5   splenocytes.

RESULTS

    CON6 Envelope Gene Design, Construction and
10 *Expression.* An artificial group M consensus *env*
gene (CON6) was constructed by generating consensus
sequences of *env* genes for each HIV-1 subtype from
sequences in the Los Alamos HIV Sequence Database,
and then generating a consensus sequence of all
15 subtype consensuses to avoid heavily sequenced
subtypes (Gaschen et al, Science 296:2354-2360
(2002), Korber et al, Science 288:1789-1796 (2000)).
Five highly variable regions from a CRF08_BC
recombinant strain (98CN006) (V1, V2, V4, V5 and a
20 region in cytoplasmic domain of gp41) were then used
to fill in the missing regions in CON6 sequence.
The CON6 V3 region is group M consensus (Figure 1A).
For high levels of expression, the codons of CON6
env gene were optimized based on codon usage for
25 highly expressed human genes (Haas et al, Curr.
Biol. 6:315-324 (2000), Andre et al, J. Virol.
72:1497-1503 (1998)). (See Fig. 1D.) The codon
optimized CON6 env gene was constructed and
subcloned into pcDNA3.1 DNA at EcoR I and BamH I
30 sites (Gao et al, AIDS Res. Hum. Retroviruses,
19:817-823 (2003)). High levels of protein

expression were confirmed with Western-blot assays
after transfection into 293T cells. To obtain
recombinant CON6 Env proteins for characterization
and use as immunogens, rVV was generated to express

5   secreted gp120 and uncleaved gp140CF (Figure 1B).
Purity for each protein was ≥90% as determined by
Coomassie blue gels under reducing conditions
(Figure 1C).

10   *CD4 Binding Domain and Other Wild-type HIV-1
Epitopes are Preserved on CON6 Proteins.* To
determine if CON6 proteins can bind to CD4 and
express other wild-type HIV-1 epitopes, the ability
of CON6 gp120 and gp140CF to bind soluble(s) CD4, to

15   bind several well-characterized anti-gp120 mabs, and
to undergo CD4-induced conformational changes was
assayed.   First, BIAcore CM5 sensor chips were
coated with either sCD4 or mabs to monitor their
binding activity to CON6 Env proteins. It was found

20   that both monomeric CON6 gp120 and oligomeric
gp140CF efficiently bound sCD4 and anti-gp120 mabs
T8, 2G12 and A32, but did not constitutively bind
mab 17b, that recognizes a CD4 inducible epitope in
the CCR5 binding site of gp120 (Figures 2A and 2B).

25   Both sCD4 and A32 can expose the 17b binding epitope
after binding to wild-type gp120 (Wyatt et al,
Nature 393;705-711 (1998), Wyatt et al, J. Virol.
69:5723-5733 (1995)). To determine if the 17b
epitope could be induced on CON6 Envs by either sCD4

30   or A32, sCD4, A32 and T8 were coated on sensor
chips, then CON6 gp120 or gp140CF captured, and mab

17b binding activity monitored. After binding sCD4
or mab A32, both CON6 gp120 and gp140CF were
triggered to undergo conformational changes and
bound mab 17b (Figures 2C and 2D). In contrast,

5   after binding mab T8, the 17b epitope was not
exposed (Figures 2C and 2D). ELISA was next used to
determine the reactivity of a panel of human mabs
against the gp120 V3 loop (447, F39F), the CD4
binding site (b12), and the gp41 neutralizing

10   determinant (2F5) to CON6 gp120 and gp140CF (Figure
2E). Both CON6 rgp120 and rgp140CF proteins bound
well to neutralizing V3 mabs 447 and F39F and to the
potent neutralizing CD4 binding site mab b12. Mab
2F5, that neutralizes HIV-1 primary isolates by

15   binding to a C-terminal gp41 epitope, also bound
well to CON6 gp140CF (Figure 2E).

       *CON6 env Gene is Biologically Functional and
       Uses CCR5 as its Coreceptor.* To determine whether

20   CON6 envelope gene is biologically functional, it
was co-transfected with the *env*-defective SG3
proviral clone into 293T cells. The pseudotyped
viruses were harvested and JC53BL cells infected.
Blue cells were detected in JC53-BL cells infected

25   with the CON6 Env pseudovirions, suggesting that
CON6 Env protein is biologically functional (Figure
3A). However, the infectious titers were 1-2 logs
lower than that of pseudovirions with either YU2 or
NL4-3 wild-type HIV-1 envelopes.

30   The co-receptor usage for the CON6 *env* gene was
next determined. When treated with CXCR4 blocking

agent AMD3100, the infectivity of NL4-3 Env-pseudovirons was blocked while the infectivity of YU2 or CON6 Env-pseudovirons was not inhibited (Figure 3B). In contrast, when treated with CCR5

5   blocking agent TAK-779, the infectivity of NL4-3 Env-pseudovirons was not affected, while the infectivity of YU2 or CON6 Env-pseudovirons was inhibited. When treated with both blocking agents, the infectivity of all pseudovirions was inhibited.

10   Taken together, these data show that the CON6 envelope uses the CCR5 co-receptor for its entry into target cells.

Reaction of CON6 gp120 With Different Subtype

15   *Sera.* To determine if multiple subtype linear epitopes are preserved on CON6 gp120, a recombinant Env protein panel (gp120 and gp140) was generated. Equal amounts of each Env protein (100 ng) were loaded on SDS-polyacrylamide gels, transferred to

20   nitrocellulose, and reacted with subtype A through G patient sera as well as anti-CON6 gp120 guinea pig sera (1:1,000 dilution) in Western blot assays. For each HIV-1 subtype, four to six patient sera were tested. One serum representative for each subtype

25   is shown in Figure 4.

It was found that whereas all subtype sera tested showed variable reactivities among Envs in the panel, all group M subtype patient sera reacted equally well with CON6 gp120 Env protein,

30   demonstrating that wild-type HIV-1 Env epitopes recognized by patient sera were well preserved on

the CON6 Env protein. A test was next made as to
whether CON6 gp120 antiserum raised in guinea pigs
could react to different subtype Env proteins. It
was found that the CON6 serum reacted to its own and
5   other subtype Env proteins equally well, with the
exception of subtype A Env protein (Figure 4).

       Induction of T Cell Responses to CON6, Subtype
       B and Subtype C Envelope Overlapping Peptides. To
10   compare T cell immune responses induced by CON6 Env
immunogens with those induced by subtype specific
immunogens, two additional groups of mice were
immunized with subtype B or subtype C DNAs and with
corresponding rVV expressing subtype B or C envelope
15   proteins. Mice immunized with subtype B (JRFL) or
subtype C (96ZM651) Env immunogen had primarily
subtype-specific T cell immune responses (Figure 5).
IFN-γ SFCs from mice immunized with JRFL (subtype B)
immunogen were detected after stimulation with
20   subtype B (MN) peptide pools, but not with either
subtype C (Chn19) or CON6 peptide pools. IFN-γ SFCs
from mice immunized with 96ZM651 (subtype C)
immunogen were detected after the stimulation with
both subtype C (Chn19) and CON6 peptide pools, but
25   not with subtype B (MN) peptide pools. In contrast,
IFN-γ SFCs were identified from mice immunized with
CON6 Env immunogens when stimulated with either CON6
peptide pools as well as by subtype B or C peptide
pools (Figure 5). The T cell immune responses
30   induced by CON6 gp140 appeared more robust than

those induced by CON6 gp120. Taken together, these
data demonstrated that CON6 gp120 and gp140CF
immunogens were capable of inducing T cell responses
that recognized T cell epitopes of wild-type subtype
5   B and C envelopes.

        Induction of Antibodies by Recombinant CON6
        gp120 and gp140CF Envelopes that Neutralize HIV-1
        Subtype B and C Primary Isolates. To determine if
10   the CON6 envelope immunogens can induce antibodies
     that neutralize HIV-1 primary isolates, guinea pigs
     were immunized with either CON6 gp120 or gp140CF
     protein. Sera collected after 4 or 5 immunizations
     were used for neutralization assays and compared to
15   the corresponding prebleed sera. Two AT-2
     inactivated HIV-1 isolates (ADA and AD8) were tested
     in syncytium inhibition assays (Table 5A). Two
     subtype B SHIV isolates, eight subtype B primary
     isolates, four subtype C, and one each subtype A, D,
20   and E primary isolates were tested in either the MT-
     2 or the luciferase-based assay (Table 5B). In the
     syncytium inhibition assay, it was found that
     antibodies induced by both CON 6 gp120 and gp140CF
     proteins strongly inhibited AT-2 inactivated ADA and
25   AD8-induced syncytia (Table 5A). In the MT-2 assay,
     weak neutralization of 1 of 2 SHIV isolates (SHIV
     SF162P3) by two gp120 and one gp140CF sera was found
     (Table 5B). In the luciferase-based assay, strong
     neutralization of 4 of 8 subtype B primary isolates
30   (BXO8, SF162, SS1196, and BAL) by all gp120 and
     gp140CF sera was found, and weak neutralization of 2

of 8 subtype B isolates (6101, 0692) by most gp120
and gp140CF sera was found. No neutralization was
detected against HIV-1 PAVO (Table 5B). Next, the
CON6 anti-gp120 and gp140CF sera were tested against
5   four subtype C HIV-1 isolates, and weak
neutralization of 3 of 4 isolates (DU179, DU368, and
S080) was found, primarily by anti-CON6 gp120 sera.
One gp140CF serum, no. 653, strongly neutralized
DU179 and weakly neutralized S080 (Table 5B).
10   Finally, anti-CON6 Env sera strongly neutralized a
subtype D isolate (93ZR001), weakly neutralized a
subtype E (CM244) isolate, and did not neutralize a
subtype A (92RW020) isolate.

Table 5A

**Ability of HIV-1 Group M Consensus Envelope CON6 Proteins to Induce
Fusion Inhibiting Antibodies**

| | | Syncytium Inhibition antibody titer[1] | |
Guinea Pig No.	Immunogen	AD8	ADA
646	gp120	270	270
647	gp120	90	90
648	gp120	90	270
649	gp120	90	90
Geometric Mean Titer		119	156
650	gp140	270	270
651	gp140	90	90
652	gp140	≥810	810
653	gp140	270	90
Geometric Mean Titer		270	207

15

[1]Reciprocal serum dilution at which HIV-induced syncytia of Sup T1 cells was
inhibited by >90% compared to pre-immune serum. All prebleed sera were negative
(titer <10).

Table 5B

Ability of Group M Consensus HIV-1 Envelope CON6 gp120 and gp140CF Proteins to Induce Antibodies that Neutralize HIV Primary Isolates

HIV Isolate (Subtype)	CON6 gp120 Protein Guinea Pig No.					CON6 gp140CF Protein Guinea Pig No.					Controls		
	646	647	648	649	GMT	650	651	652	653	GMT	TriMab‡	CD4-IgG2	HIV+ Serum
SHIV 89.6P*(B)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	NT	NT	NT
SHIV SF162P3*(B)	<20	30	48	<20	<20	27	<20	<20	<20	<20	NT	0.2µg/ml	NT
BX08(B)	270	183	254	55	102	199	64	229	150	187	0.7µg/ml	NT	236#
6101(B)	<20	38	35	<20	<20	<20	90	72	73	39	1.1µg/ml	NT	NT
BG1168(B)	<20	<20	<20	<20	<20	40	<20	<20	25	<20	2.7µg/ml	NT	NT
0692(B)	31	32	34	24	24	28	33	30	45	33	0.8µg/ml	NT	769
PAVO(B)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	2.9µg/ml	NT	NT
SF162(B)	2,146	308	110	282	379	206	5,502	15,098	174	1,313	NT	NT	>540
SS1196(B)	206	26	148	59	83	381	401	333	81	253	NT	NT	301#
BAL(B)	123	90	107	138	113	107	146	136	85	116	NT	NT	3307
92RW020(A)	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	NT	NT	693
DU179(C)	<20	43	<20	24	<20	<20	<20	24	515	33	NT	0.8µg/ml	NT
DU368(C)	25	35	62	<20	27	<20	<20	<20	23	<20	NT	2.3µg/ml	NT
S021(C)	<20	<20	33	<20	<20	<20	<20	<20	<20	<20	NT	8.3µg/ml	NT
S080(C)	24	37	70	41	40	<20	<20	<20	52	<20	NT	3.4µg/ml	NT
93ZR001(D)	275	144	126	114	154	306	195	129	173	191	NT	NT	693
CM244(E)	35	43	64	ND	46	31	25	27	25	26	NT	NT	693

*MT-2 Assay; All other HIV isolates were tested in the M7-luciferase assay.
HIV-1 isolates QH0692, SS1196, SF162, 6101, BX08, BG1168, BAL were assayed with post-injection 5 serum; other HIV-1 isolates were assayed with post-injection 4 serum. ND = not done.
HIV+ sera was either HIV-1+ human serum (LEH3) or an anti-gp120 guinea pig serum (#) with known neutralizing activity for HIV-1 isolate SS1196. GMT = geometric mean titer of four animals per group. Neutralizing titers reported are after subtraction of any background neutralization in prebleed sera.
‡TriMab₂ = a mixture of human mabs 2F5, b12, 2G12.

CONCLUSIONS

        The production of an artificial HIV-1 Group M
consensus env genes (encoding sequences) (CON6 and
5   Con-S) have been described that encodes a functional
Env protein that is capable of utilizing the CCR5
co-receptor for mediating viral entry. Importantly,
these Group M consensus envelope genes could induce
T and B cell responses that recognized epitopes of
10 subtype B and C HIV-1 primary isolates. In
addition, Con-S induces antibodies that strongly
neutralize Subtype-C and A HIV-1 strains (see
Table 3).

        The correlates of protection to HIV-1 are not
15 conclusively known. Considerable data from animal
models and studies in HIV-1-infected patients
suggest the goal of HIV-1 vaccine development should
be the induction of broadly-reactive CD4+ and CD8+
anti-HIV-1 T cell responses (Letvin et al, Annu.
20 Rev. Immunol. 20:73-99 (2002)) and high levels of
antibodies that neutralize HIV-1 primary isolates of
multiple subtypes (Mascola et al, J. Virol. 73:4009-
4018 (1999), Mascola et al, Nat. Med. 6:270-210
(2000)).

25   The high level of genetic variability of HIV-1
has made it difficult to design immunogens capable
of inducing immune responses of sufficient breadth
to be clinically useful. Epitope based vaccines for
T and B cell responses (McMichael et al, Vaccine
30 20:1918-1921 (2002), Sbai et al, Curr. Drug Targets
Infect, Disord. 1:303-313 (2001), Haynes, Lancet

73

348:933-937 (1996)), constrained envelopes
reflective of fusion intermediates (Fouts et al,
Proc. Natl. Acad. Sci. USA 99:11842-22847 (2002)),
as well as exposure of conserved high-order

5   structures for induction of anti-HIV-1 neutralizing
antibodies have been proposed to overcome HIV-1
variability (Roben et al, J. Virol. 68:4821-4828
(1994), Saphire et al, Science 293:1155-1159
(2001)). However, with the ever-increasing

10   diversity and rapid evolution of HIV-1, the virus is
a rapidly moving complex target, and the extent of
complexity of HIV-1 variation makes all of these
approaches problematic. The current most common
approach to HIV-1 immunogen design is to choose a

15   wild-type field HIV-1 isolate that may or may not be
from the region in which the vaccine is to be
tested. Polyvalent envelope immunogens have been
designed incorporating multiple envelope immunogens
(Bartlett et al, AIDS 12:1291-1300 (1998), Cho et

20   al, J. Virol. 75:2224-2234 (2001)).

     The above-described study tests a new strategy
for HIV-1 immunogen design by generating a group M
consensus *env* gene (CON6) with decreased genetic
distance between this candidate immunogen and wild-

25   type field virus strains. The CON6 *env* gene was
generated for all subtypes by choosing the most
common amino acids at most positions (Gaschen et al,
Science 296:2354-2360 (2002), Korber et al, Science
288:1789-1796 (2000)). Since only the most common

30   amino acids were used, the majority of antibody and
T cell epitopes were well preserved. Importantly,

the genetic distances between the group M consensus
env sequence and any subtype *env* sequences was about
15%, which is only half of that between wild-type
subtypes (30%) (Gaschen et al, Science 296:2354-2360
5   (2002)). This distance is approximately the same as
that among viruses within the same subtype.
Further, the group M consensus *env* gene was also
about 15% divergent from any recombinant viral *env*
gene, as well, since CRFs do not increase the
10   overall genetic divergence among subtypes.

Infectivity of CON6-Env pseudovirions was
confirmed using a single-round infection system,
although the infectivity was compromised, indicating
the artificial envelope was not in an "optimal"
15   functional conformation, but yet was able to mediate
virus entry. That the CON6 envelope used CCR5 (R5)
as its coreceptor is important, since majority of
HIV-1 infected patients are initially infected with
R5 viruses.

20   BIAcore analysis showed that both CON6 gp120
and gp140CF bound sCD4 and a number of mabs that
bind to wild-type HIV-1 Env proteins. The
expression of the CON6 gp120 and 140CF proteins that
are similar antigenically to wild-type HIV-1
25   envelopes is an important step in HIV-1 immunogen
development. However, many wild-type envelope
proteins express the epitopes to which potent
neutralizing human mabs bind, yet when used as
immunogens themselves, do not induce broadly
30   neutralizing anti-HIV-1 antibodies of the
specificity of the neutralizing human mabs.

The neutralizing antibody studies were encouraging in that both CON6 gp120, CON6 gp140CF and Con-S gp140CFI induced antibodies that neutralized select subtype B, C and D HIV-1 primary

5   isolates, with Con-S gp140CFI inducing the most robust neutralization of non-subtype B primary HIV isolates. However, it is clear that the most difficult-to-neutralize primary isolates (PAVO, 6101, BG1168, 92RW020, CM244) were either only

10 weakly or not neutralized by anti-CON6 gp120 or gp140 sera (Table 4b). Nonetheless, the Con-S envelope immunogenicity for induction of neutralizing antibodies is promising, given the breadth of responses generated with the Con-S

15 subunit gp140CFI envelope protein for non-subtype B HIV isolates. Previous studies with poxvirus constructs expressing gp120 and gp160 have not generated high levels of neutralizing antibodies (Evans et al, J. Infect. Dis. 180:290-298 (1999),

20 Polacino et al, J. Virol. 73:618-630 (1999), Ourmanov et al, J. Virol. 74:2960-2965 (2000), Pal et al, J. Virol 76:292-302 (2002), Excler and Plotkin, AIDS 11(Suppl A):S127-137 (1997). rVV expressing secreted CON6 gp120 and gp140 have been

25 constructed and antibodies that neutralize HIV-1 primary isolates induced. An HIV neutralizing antibody immunogen can be a combination of Con-S gp140CFI, or subunit thereof, with immunogens that neutralize most subtype B isolates.

The structure of an oligomeric gp140 protein is critical when evaluating protein immunogenicity. In this regard, study of purified CON6 gp140CF proteins by fast performance liquid chromatography (FPLC) and analytical ultracentrifiguration has demonstrated that the purified gp140 peak consists predominantly of trimers with a small component of dimers.

Thus, centralized envelopes such as CON6, Con-S or 2003 group M or subtype consensus or ancestral encoding sequences described herein, are attractive candidates for preparation of various potentially "enhanced" envelope immunogens including CD4-Env complexes, constrained envelope structures, and trimeric oligomeric forms. The ability of CON6-induced T and B cell responses to protect against HIV-1 infection and/or disease in SHIV challenge models will be studied in non-human primates.

The above study has demonstrated that artificial centralized HIV-1 genes such as group M consensus *env* gene (CON6) and Con-S can also induce T cell responses to T cell epitopes in wild-type subtype B and C Env proteins as well as to those on group M consensus Env proteins (Figure 5). While the DNA prime and rVV boost regimen with CON6 gp140CF immunogen clearly induced IFN-γ producing T cells that recognized subtype B and C epitopes, further studies are needed to determine if centralized sequences such as are found in the CON6 envelope are significantly better at inducing cross-

clade T cell responses than wild-type HIV-1 genes
(Ferrari et al, Proc. Natl. Acad. Sci. USA 94:1396-
1401 (1997), Ferrari et al, AIDS Res. Hum.
Retroviruses 16:1433-1443 (2000)). However, the

5   fact that CON6 (and Con-S, env encoding sequence)
prime and boosted splenocyte T cells recognized HIV-
1 subtype B and C T cell epitopes is an important
step in demonstration that CON6 (and Con-S) can
induce T cell responses that might be clinically

10   useful.

       Three computer models (consensus, ancestor and
center of the tree (COT)) have been proposed to
generate centralized HIV-1 genes (Gaschen et al,
Science 296:2354-2360 (2002), Gao et al, Science

15   299:1517-1518 (2003), Nickle et al, Science
299:1515-1517 (2003), Korber et al, Science
288:1789-1796 (2000). They all tend to locate at
the roots of the star-like phylogenetic trees for
most HIV-1 sequences within or between subtypes. As

20   experimental vaccines, they all can reduce the
genetic distances between immunogens and field virus
strains. However, consensus, ancestral and COT
sequences each have advantages and disadvantages
(Gaschen et al, Science 296:2354-2360 (2002), Gao et

25   al, Science 299:1517-1518 (2003), Nickle et al,
Science 299:1515-1517 (2003). Consensus and COT
represent the sequences or epitopes in sampled
current wild-type viruses and are less affected by
outliers HIV-1 sequences, while ancestor represents

30   ancestral sequences that can be significantly
affected by outlier sequences. However, at present,

it is not known which centralized sequence can serve as the best immunogen to elicit broad immune responses against diverse HIV-1 strains, and studies are in progress to test these different strategies.

5   Taken together, the data have shown that the HIV-1 artificial CON6 and Con-S envelope can induce T cell responses to wild-type HIV-1 epitopes, and can induce antibodies that neutralize HIV-1 primary isolates, thus demonstrating the feasibility and

10   promise of using artificial centralized HIV-1 sequences in HIV-1 vaccine design.

EXAMPLE 2

HIV-1 Subtype C Ancestral and Consensus Envelope
15   Glycoproteins

EXPERIMENTAL DETAILS

HIV-1 subtype C ancestral and consensus *env* genes were obtained from the Los Alamos HIV Molecular Immunology Database (http://hiv-

20   web.lanl.gov/immunology), codon-usage optimized for mammalian cell expression, and synthesized (Fig. 6). To ensure optimal expression, a Kozak sequence (GCCGCCGCC) was inserted immediately upstream of the initiation codon. In addition to the full-length

25   genes, two truncated *env* genes were generated by introducing stop codons immediately after the gp41 membrane-spanning domain (IVNR) and the gp120/gp41 cleavage site (REKR), generating gp140 and gp120 form of the glycoproteins, respectively (Fig. 8).

Genes were tested for integrity in an *in vitro* transcription/translation system and expressed in mammalian cells. To determine if the ancestral and consensus subtype C envelopes were capable of

5   mediating fusion and entry, *gp160* and *gp140* genes were co-transfected with an HIV-1/SG3Δ*env* provirus and the resulting pseudovirions tested for infectivity using the JC53-BL cell assay (Fig. 7). Co-receptor usage and envelope neutralization

10   sensitivity were also determined with slight modifications of the JC53-BL assay. Codon-usage optimized and rev-dependent 96ZAM651 *env* genes were used as contemporary subtype C controls.

RESULTS

15

Codon-optimized subtype C ancestral and consensus envelope genes (*gp160, gp140, gp120*) express high levels of *env* glycoprotein in mammalian cells (Fig. 9).

20   Codon-optimized subtype C *gp160* and *gp140* glycoproteins are efficiently incorporated into virus particles. Western Blot analysis of sucrose-purified pseudovirions reveals ten-fold higher levels of virion incorporation of the codon-

25   optimized envelopes compared to that of a rev-dependent contemporary envelope controls (Fig. 10A).

Virions pseudotyped with either the subtype C consensus *gp160* or *gp140* envelope were more infectious than pseudovirions containing the

30   corresponding gp160 and gp140 ancestral envelopes.

Additionally, *gp160* envelopes were consistently more infectious than their respective *gp140* counterparts (Fig. 10B).

5   Both subtype C ancestral and consensus envelopes utilize CCR5 as a co-receptor to mediate virus entry (Fig. 11).

The infectivity of subtype C ancestral and consensus *gp160* containing pseudovirions was neutralized by plasma from subtype C infected

10   patients. This suggests that these artificial envelopes possess a structure that is similar to that of native HIV-1 *env* glycoproteins and that common neutralization epitopes are conserved. No significant differences in neutralization potential

15   were noted between subtype C ancestral and consensus *env* glycoproteins (gp160) (Fig. 12).

CONCLUSIONS

HIV-1 subtype C viruses are among the most prevalent circulating isolates, representing

20   approximately fifty percent of new infections worldwide. Genetic diversity among globally circulating HIV-1 strains poses a challenge for vaccine design. Although HIV-1 Env protein is highly variable, it can induce both humoral and cellular

25   immune responses in the infected host. By analyzing 70 HIV-1 complete subtype C *env* sequences, consensus and ancestral subtype C *env* genes have been generated. Both sequences are roughly equidistant from contemporary subtype C strains and thus

expected to induce better cross-protective immunity.
A reconstructed ancestral or consensus sequence
derived-immunogen minimizes the extent of genetic
differences between the vaccine candidate and

5   contemporary isolates. However, consensus and
ancestral subtype C env genes differ by 5% amino
acid sequences. Both consensus and ancestral
sequences have been synthesized for analyses.
Codon-optimized subtype C ancestral and consensus

10   envelope genes have been constructed and the in
vitro biological properties of the expressed
glycoproteins determined. Synthetic subtype C
consensus and ancestral env genes express
glycoproteins that are similar in their structure,

15   function and antigenicity to contemporary subtype C
wild-type envelope glycoproteins.

EXAMPLE 3

Codon-Usage Optimization of Consensus of Subtype C

20   gag and nef Genes (C.con.gag and C.con.nef)

Subtype C viruses have become the most
prevalent viruses among all subtypes of Group M
viruses in the world. More than 50% of HIV-1

25   infected people are currently carrying HIV-1 subtype
C viruses. In addition, there is considerable
intra-subtype C variability: different subtype C
viruses can differ by as much as 10%, 6%, 17% and

16% of their Gag, Pol, Env and Nef proteins,
respectively. Most importantly, the subtype C
viruses from one country can vary as much as the
viruses isolated from other parts of the world. The
5   only exceptions are HIV-1 strains from India/China,
Brazil and Ethiopia/Djibouti where subtype C appears
to have been introduced more recently. Due to the
high genetic variability of subtype C viruses even
within a single country, an immunogen based on a
10   single virus isolate may not elicit protective
immunity against other isolates circulating in the
same area.

Thus *gag* and *nef* gene sequences of subtype C
viruses were gathered to generate consensus
15   sequences for both genes by using a 50% consensus
threshold. To avoid a potential bias toward founder
viruses, only one sequence was used from
India/China, Brazil and Ethiopia/Djibouti,
respectively, to generate the subtype C consensus
20   sequences (C.con.gag and C.con.nef). The codons of
both C.con.gag and C.con.nef genes were optimized
based on the codon usage of highly expressed human
genes. The protein expression following transfection
into 293T cells is shown in Figure 13. As can be
25   seen, both consensus subtype C Gag and Nef proteins
were expressed efficiently and recognized by Gag-
and Nef-specific antibodies. The protein expression
levels of both C.con.gag and C.con.nef genes are
comparible to that of native subtype *env* gene
30   (96ZM651).

EXAMPLE 4

Synthesis of a Full Length "*Consensus of the*
Consensus env Gene with Consensus Variable Regions"
(CON-S)

In the synthesized "consensus of the consensus"
env gene (CON6), the variable regions were replaced
with the corresponding regions from a contemporary
subtype C virus (98CN006). A further con/con gene
has been designed that also has consensus variable
regions (CON-s). The codons, of the Con-S *env* gene
were optimized based on the codon usage of highly
expressed human genes. (See Figs. 14A and 14B for
amino acid sequences and nucleic acid sequences,
respectfully.)

Paired oligonucleotides (80-mers) which overlap
by 20 bp at their 3' ends and contain invariant
sequences at their 5' and 3' ends, including the
restriction enzyme sites EcoRI and BbsI as well as
BsmBI and BamHI, respectively, were designed. BbsI
and BamHI are Type II restriction enzymes that
cleave outside of their recognition sequences. They
have been positioned in the oligomers in such a way
that they cleave the first four resides adjacent to
the 18 bp invariant region, leaving 4 base 5'
overhangs at the end of each fragment for the
following ligation step. 26 paired oligomers were
linked individually using PCR and primers
complimentary to the 18 bp invariant sequences.

Each pair was cloned into pGEM-T (Promega) using the
T/A cloning method and sequenced to confirm the
absence of inadvertent mutations/deletions. pGEM-T
subclones containing the proper inserts were then

5   digested, run on a 1% agarose gel, and gel purified
(Qiagen). Four individual 108-mers were ligated
into pcDNA3.1 (Invitrogen) in a multi-fragment
ligation reaction. The four-way ligations occurred
among groups of fragments in a stepwise manner from

10   the 5' to the 3' end of the gene. This process was
repeated until the entire gene was reconstructed in
the pcDNA3.1 vector.

A complete Con-S gene was constructed by
ligating the codon usage optimized oligo pairs

15   together. To confirm its open reading frame, an *in
vitro* transcription and translation assay was
performed. Protein products were labeled by S^{35}-
methionine during the translation step, separated on
a 10% SDS-PAGE, and detected by radioautography.

20   Expected size of the expressed Con-S gp160 was
identified in 4 out of 7 clones (Fig. 14C).

CONs Env protein expression in the mammalian
cells after transfected into 293T cells using a
Western blot assay (Figure 15). The expression level

25   of Con-S Env protein is very similar to what was
observed from the previous CON6 *env* clone that
contains the consensus conservative regions and
variable loops from 98CN006 virus isolate.

The Env-pseudovirons was produced by

30   cotransfecting Con-S *env* clone and *env*-deficient SG3

proviral clone into 293T cells. Two days after
transfection, the pseudovirions were harvested and
infected into JC53BL-13 cells. The infectious units
(IU) were determined by counting the blue cells
5   after staining with X-gal in three independent
experiments. When compared with CON6 *env* clone, Con-
S *env* clones produce similar number of IU in JC53BL-
13 cells (Figure 16). The IU titers for both are
about 3 log higher than the SG3 backbone clone
10   control (No Env). However, the titers are also
about 2 log lower than the positive control (the
native HIV-1 *env* gene, NL4-3 or YU2). These data
suggest that both *consensus* group M *env* clones are
biologically functional. Their functionality,
15   however, has been compromised. The functional
consensus *env* genes indicate that these Env proteins
fold correctly, preserve the basic conformation of
the native Env proteins, and are able to be
developed as universal Env immunogens.

20   It was next determined what coreceptor Con-S
Env uses for its entry into JC53-BL cells. When
treated with CXCR4 blocking agent AMD3100, the
infectivity of NL4-3 Env-pseudovirons was blocked
while the infectivity of YU2, Con-S or CON6 Env-
25   pseudovirons was not inhibited. In contrast, when
treated with CCR5 blocking agent TAK779, the
infectivity of NL4-3 Env-pseudovirons was not
affected, while the infectivity of YU2, Con-S or
CON6 Env-pseudovirons was inhibited. When treated
30   with both blocking agents, the infectivity of all
pseudovirions was inhibited. Taken together, these

data show that the Con-S as well as CON6 envelope
uses the CCR5 but not CXCR4 co-receptor for its
entry into target cells.

It was next determined whether CON6 or Con-S
5   Env proteins could be equally efficiently
incorporated in to the pseudovirions. To be able
precisely compare how much Env proteins were
incorporated into the pseudovirions, each
pseudovirions is loaded on SDS-PAGE at the same
10   concentraion: 5µg total protein for cell lysate,
25ng p24 for cell culture supernatant, or 150ng p24
for purified virus stock (concentrated pseudovirions
after super-speed centrifugation). There was no
difference in amounts of Env proteins incorporated
15   in CON6 or Con-S Env-pseudovirions in any
preparations (cell lysate, cell culture supernatant
or purified virus stock) (Figure 17).

<u>EXAMPLE 5</u>

Synthesis of a *Consensus* Subtype A Full Length *env*
20   (A.con.env) Gene

Subtype A viruses are the second most prevalent
HIV-1 in the African continent where over 70% of
HIV-1 infections have been documented. Consensus
25   *gag, env* and *nef* genes for subtype C viruses that
are the most prevalent viruses in Africa and in the
world were previously generated. Since genetic
distances between subtype A and C viruses are as
high as 30% in the *env* gene, the cross reactivity or
30   protection between both subtypes will not be

optimal. Two group M consensus *env* genes for all
subtypes were also generated. However, to target
any particular subtype viruses, the subtype specific
consensus genes will be more effective since the

5   genetic distances between subtype consensus genes
and field viruses from the same subtype will be
smaller than that between group M consensus genes
and these same viruses. Therefore, consensus genes
need to be generated for development of subtype A

10   specific immunogens. The codons of the A.con.env
gene were optimized based on the codon usage of
highly expressed human genes. (See Figs. 18A and
18B for amino acid and nucleic acid sequences,
respectively.)

15   Each pair of the oligos has been amplified,
cloned, ligated and sequenced. After the open
reading frame of the A.con *env* gene was confirmed by
an *in vitro* transcription and translation system,
the A.con *env* gene was transfected into the 293T

20   cells and the protein expression and specificity
confirmed with the Western blot assay (Figure 18).
It was then determined whether A.con envelope is
biologically functional. It was co-transfected with
the *env*-defective SG3 proviral clone into 293T

25   cells. The pseudotyped viruses were harvested and
used to infect JC53BL cells. Blue cells were
detected in JC53-BL cells infected with the A.con
Env-pseudovirions, suggesting that A.con Env protein
is biologically functional (Table 6). However, the

30   infectious titer of A.con Env-psuedovirions was
about 7-fold lower than that of pseudovirions with

wild-type subtype C envelope (Table 6). Taken
together, the biological function A.con Env proteins
suggests that it folds correctly and may induce
linear and conformational T and B cell epitopes if
5   used as an Env immunogen.

JC53BL13 (IU/ul)

	3/31/03	4/7/03	4/25/03
	non filtered supt.	0.22μm filtered	0.22μm filtered
A.con +SG3	4	8.5	15.3
96ZM651 +SG3	87	133	104
SG3 backbone	0	0.07	0.03
Neg control	0	0.007	0

Table 6. Infectivity of pseudovirons with A.con env genes

EXAMPLE 6

10   Design of Full Length "Consensus of the Consensus
gag, pol and nef Genes" (M.con.gag, M.con.pol and
M.con.nef) and a Subtype C Consensus pol Gene
(C.con.pol)

15   For the group M consensus genes, two different
env genes were constructed, one with virus specific
variable regions (CON6) and one with consensus
variable regions (Con-S). However, analysis of T
cell immune responses in immunized or vaccinated
20   animals and humans shows that the env gene normally
is not a main target for T cell immune response

although it is the only gene that will induce
neutralizing antibody. Instead, HIV-1 Gag, Pol and
Nef proteins are found to be important for inducing
potent T cell immune responses. To generate a

5   repertoire of immunogens that can induce both
broader humoral and cellular immune responses for
all subtypes, it may be necessary to construct other
group M consensus genes other than *env* gene alone.
"Consensus of the consensus" *gag*, *pol* and *nef* genes

10 (M.con.gag., M.con.pol and M.con.nef) have been
designed. To generate a subtype consensus pol gene,
the subtype C consensus *pol* gene (C.con.pol) was
also designed. The codons of the M.con.gag.,
M.con.pol, M.con.nef and C.con.pol. genes were

15 optimized based on the codon usage of highly
expressed human genes. (See Fig. 19 for nucleic
acid and amino acid sequences.)

<center>EXAMPLE 7</center>

<center>Synthetic Subtype B Consensus *gag* and *env* Genes</center>

20 EXPERIMENTAL DETAILS

Subtype B consensus *gag* and *env* sequences were
derived from 37 and 137 contemporary HIV-1 strains,
respectively, codon-usage optimized for mammalian
cell expression, and synthesized (Figs. 20A and

25 20B). To ensure optimal expression, a Kozak
sequence (GCCGCCGCC) was inserted immediately
upstream of the initiation codon. In addition to
the full-length *env* gene, a truncated *env* gene was
generated by introducing a stop codon immediately

<center>90</center>

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after the gp41 membrane-spanning domain (IVNR) to
create a *gp145* gene. Genes were tested for
integrity in an *in vitro* transcription/translation
system and expressed in mammalian cells. (Subtype B

5 consensus Gag and Env sequences are set forth in
Figs. 20C and 20D, respectively.)

    To determine if the subtype B consensus
envelopes were capable of mediating fusion and
entry, *gp160* and *gp145* genes were co-transfected

10 with an HIV-1/SG3Δenv provirus and the resulting
pseudovirions were tested for infectivity using the
JC53-BL cell assay. JC53-BL cells are a derivative
of HeLa cells that express high levels of CD4 and
the HIV-1 coreceptors CCR5 and CXCR4. They also

15 contain the reporter cassettes of luciferase and β-
galactosidase that are each expressed from an HIV-1
LTR. Expression of the reporter genes is dependent
on production of HIV-1 Tat. Briefly, cells are
seeded into 24-well plates, incubated at 37°C for 24

20 hours and treated with DEAE-Dextran at 37°C for
30min. Virus is serially diluted in 1% DMEM, added
to the cells incubating in DEAE-dextran, and allowed
to incubate for 3 hours at 37°C after which an
additional 500μL of cell media is added to each

25 well. Following a final 48-hour incubation at 37°C,
cells are fixed, stained using X-Gal, and overlaid
with PBS for microscopic counting of blue foci.
Counts for mock-infected wells, used to determine
background, are subtracted from counts for the

30 sample wells. Co-receptor usage and envelope

neutralization sensitivity were also determined with
slight modifications of the JC53-BL assay.

To determine whether the subtype B consensus
Gag protein was capable of producing virus-like
5   particles (VLPs) that incorporated Env
glycoproteins, 293T cells were co-transfected with
subtype B consensus *gag* and *env* genes. 48-hours
post-transfection, cell supernatants containing VLPs
were collected, clarified in a tabletop centrifuge,
10   filtered through a 0.2mM filter, and pellet through
a 20% sucrose cushion. The VLP pellet was
resuspended in PBS and transferred onto a 20-60%
continuous sucrose gradient. Following overnight
centrifugation at 100,000 x g, 0.5 ml fractions were
15   collected and assayed for p24 content. The
refractive index of each fraction was also measured.
Fractions with the correct density for VLPs and
containing the highest levels of p24 were pooled and
pellet a final time. VLP-containing pellets were
20   re-suspended in PBS and loaded on a 4-20% SDS-PAGE
gel. Proteins were transferred to a PVDF membrane
and probed with serum from a subtype B HIV-1
infected individual.

RESULTS

25

Codon-usage optimized, subtype B consensus
envelope (*gp160, gp145*) and *gag* genes express high
levels of glycoprotein in mammalian cells (Fig. 21).
Subtype B gp160 and gp145 glycoproteins are
30   efficiently incorporated into virus particles.

Western Blot analysis of sucrose-purified

pseudovirions suggests at least five-fold higher

levels of consensus B envelope incorporation

compared to incorporation of a rev-dependent

5   contemporary envelope (Fig.23A). Virions

pseudotyped with either the subtype B consensus

gp160 or gp145 envelope are more infectious than

pseudovirions containing a rev-dependent

contemporary envelope (Fig. 23 B).

10   Subtype B consensus envelopes utilize CCR5 as

the co-receptor to gain entry into CD4 bearing

target cells (Fig. 22).

The infectivity of pseudovirions containing the

subtype B consensus gp160 envelope was neutralized

15   by plasma from HIV-1 subtype B infected patients

(Fig. 24C) and neutralizing monoclonal antibodies

(Fig. 24A). This suggests that the subtype B

synthetic consensus B envelopes is similar to native

HIV-1 Env glycoproteins in its overall structure and

20   that common neutralization epitopes remain intact.

Figs. 24B and 24D show neutralization profiles of a

subtype B control envelope (NL4.3 Env).

Subtype B consensus Gag proteins are able to

bud from the cell membrane and form virus-like

25   particles (Fig. 25A). Co-transfection of the codon-

optimized subtype B consensus *gag* and *gp160* genes

produces VLPs with incorporated envelope (Fig. 25B).

CONCLUSIONS

    The synthetic subtype B consensus *env* and *gag*
genes express viral proteins that are similar in
their structure, function and antigenicity to
5   contemporary subtype B Env and Gag proteins. It is
contemplated that immunogens based on subtype B
consensus genes will elicit CTL and neutralizing
immune responses that are protective against a broad
set of HIV-1 isolates.

10   *   *   *

    All documents and other information sources
cited above are hereby incorporated in their
entirety by reference. Also incorporated by
reference is Liao et al, J. Virol. 78:5270 (2004)).